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(54) Title: IMMUNOGENIC COMPOSITIONS AGAINST HELICOBACTER INFECTION, POLYPEPTIDES FOR USE IN THE COMPOSITIONS, AND NUCLEIC ACID SEQUENCES ENCODING SAID POLYPEPTIDES			
(57) Abstract			
<p>There is provided an immunogenic composition capable of inducing protective antibodies against <i>Helicobacter</i> infection characterized in that it comprises: i) at least one sub-unit of a urease structural polypeptide from <i>Helicobacter pylori</i>, or a fragment thereof, said fragment being recognized by antibodies reacting with <i>Helicobacter felis</i> urease, and/or at least one sub-unit of a urease structural polypeptide from <i>Helicobacter felis</i>, or a fragment thereof, said fragment being recognized by antibodies reacting with <i>Helicobacter pylori</i> urease; ii) and/or, a heat shock protein (Hsp), or chaperonin, from <i>Helicobacter</i>, or a fragment of said protein. The preparation, by recombinant means, of such immunogenic composition is also provided.</p>			

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IMMUNOGENIC COMPOSITIONS AGAINST HELICOBACTER
INFECTION, POLYPEPTIDES FOR USE IN THE COMPOSITIONS,
AND NUCLEIC ACID SEQUENCES ENCODING SAID POLYPEPTIDES

Technical Field

The present invention relates to immunogenic compositions for inducing protective antibodies against *Helicobacter spp.* infection. It also relates to proteinaceous material derived from *Helicobacter*, and to nucleic acid sequences encoding them. Antibodies to these proteinaceous materials are also included in the invention.

Background Art

H. pylori is a microorganism, which infects human gastric mucosa and is associated with active chronic gastritis. It has been shown to be an aetiological agent in gastroduodenal ulceration (Peterson, 1991), and two recent studies have reported that persons infected with *H. pylori* had a higher risk of developing gastric cancer (Nomura et al., 1991; Parsonnet et al., 1991).

In vivo studies of the bacterium, and consequently, work on the development of appropriate preventive or therapeutic agents, has been severely hindered by the fact that *Helicobacter pylori* only associates with gastric-type epithelium from very few animal hosts, none of which are suitable for use as laboratory models.

A mouse model of gastric colonization has been developed using a helical bacterium isolated from cat gastric mucus (Lee et al., 1988, 1990) and identified as a member of the genus *Helicobacter*. It has been named *H. felis* (Paster et al., 1990).

To date, only limited information concerning *H. felis* and the extent of its similarities and differences with *H. pylori* is available. The reliability of the mouse

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model for the development of treatments for *H. pylori* infection is, therefore, uncertain. Recently, it was shown that *H. pylori* urease is a protective antigen in the *H. felis*/mouse model (Davin et al., 1993; Cortesy-Theulaz et al., 1993).

It is, therefore, an aim of the present invention to provide therapeutic and preventive compositions for use in *Helicobacter* infection, which furthermore can be tested in laboratory animals.

It is known that *H. pylori* expresses urease activity and that urease plays an important role in bacterial colonization and mediation of certain pathogenic processes (Ferrero and Lee, 1991; Hazel et al., 1991).

The genes coding for the urease structural polypeptides of *H. pylori* (*UreA*, *UreB*) have been cloned and sequenced (Labigne et al., 1991; and French Patent Application FR 8813135), as have the genes coding the "accessory" polypeptides necessary for urease activity in *H. pylori* (International patent application WO 93/07273).

Attempts have been made to use nucleic acid sequences from the *H. pylori* urease gene cluster as probes to identify urease sequences in *H. felis*. However, none of these attempts have been successful. Furthermore, the establishment and maintenance of *H. felis* cultures *in vitro* is extremely difficult, and the large quantities of nucleases present in the bacteria complicates the extraction of DNA.

Disclosure of the Invention

The present inventors have, however, succeeded in cloning and sequencing the genes of the urease structural polypeptides of *H. felis*, and of the accessory polypeptides. This has enabled, in the context of the invention, the comparison of the amino acid sequence data for the *H. felis Ure* gene products with that for *Helicobacter pylori*, and a high degree of conservation between the urease sub-units has been found. An immunological relationship between the

two ureases exists, and protective antibodies to *Helicobacter* infection can be induced using the urease sub-units or fragments thereof as immunogens.

Indeed, to elucidate the efficiency of individual urease subunits to act as mucosal immunogens, the genes encoding the respective urease sub-units (*UreA* and *UreB*) of *Helicobacter pylori* and *Helicobacter felis* have been cloned in an expression vector (pMAL) and expressed in *Escherichia coli* cells as translational fusion proteins. The recombinant *UreA* and *UreB* proteins have been purified by affinity and anion exchange chromatography techniques, and have predicted molecular weights of approximately 68 and 103 kDa, respectively.

Western blotting studies indicated that the urease components of the fusion proteins are strongly immunogenic and are specifically recognized by polyclonal rabbit anti-*Helicobacter* sera. Orogastric immunization of mice with 50 μ m of recombinant *H. felis* *UreB*, administered in combination with a mucosal adjuvant (cholera toxin), protected 60% ($n = 7$; $p < 0.005$) of mice from gastric colonization by *H. felis* bacteria at over 4 months. This compared with a value of 25% ($n = 8$; $p > 0.05$) for the heterologous *H. pylori* *UreB* antigen. For the first time, a recombinant subunit antigen has been shown to induce an immunoprotective response against gastric *Helicobacter* infection.

The inventors have also identified, in the context of the invention, new heat shock proteins or chaperonins in *Helicobacter*, which have an enhancing effect on urease activity. Use of the chaperonins in an immunogenic composition may induce therefore an enhancement of protection.

Indeed, the genes encoding each of the HspA and HspB polypeptides of *Helicobacter pylori* have been cloned, expressed independently as fused proteins to the Maltose-Binding-Protein (MBP), and purified on a large scale. These proteins have been used as recombinant antigens to immunize rabbits, and in Western immunoblotting assays as well as ELISA, to determine their immunogenicity in patients infected with HP (HP+). The MBP-HspA and MBP-

HspB fusion proteins have been shown to retain their antigenic properties. Comparison of the humoral immune response against HspA and/or HspB in (HP-) patient sera demonstrated that not only HspB but also HspA was recognized by (HP+) patient sera (29/38 and 15/38, respectively). None of the 14 uninfected patients had antibodies reacting with the Hsps.

Brief Description Of The Invention

This invention will be described in greater detail by reference to the following drawings:

Fig. 1. Transposon mutagenesis and sequencing of pILL205. Linear restriction maps of recombinant cosmid pILL199 and recombinant plasmid pILL205 (and the respective scale markers) are presented. Numbers in parentheses indicate the sizes of *H. felis* DNA fragments inserted into one of the cloning vectors (pILL575 described in J. Bact. 1991, 173:1920-1931 or pILL570, described in Res. Microb. 1992, 143:1526, respectively). The "plus" and "minus" signs within circles correspond to the insertion sites of the MiniTn3-Km transposon in pILL205; "plus" signs indicate that the transposon did not inactivate urease expression, whereas negative signs indicate that urease expression was abolished. The letters refer to mutant clones, which were further characterized for quantitative urease activity and for the synthesis of urease gene products. The location of the structural urease genes (*UreA* and *UreB*) on pILL205 are represented by boxes, the lengths of which are proportional to the sizes of the respective open-reading frames. The arrows refer to the orientation of transcription. The scale at the bottom of the Figure indicates the sizes (in kilobases) of the *Hind*III and *Pst*I restriction fragments. Restriction sites are represented as follows: B, *Bam*HI; Pv, *Pvu*II; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; C, *Cla*I; Ps, *Pst*I. Letters within parentheses indicate that the sites originated from the cloning vector.

Fig. 2. Western blot analysis of whole-cell extracts of *E. coli* HB101 cells harboring recombinant plasmids were reacted with rabbit polyclonal antiserum (diluted 1:1, 1000) raised against *H. felis* bacteria.

A) Extracts were of *E. coli* cells harboring: plasmid vector pILL570 (lane 1); recombinant plasmid pILL205, described in Molec. Microb. 1993, 9:323-333 (lane 2); and pILL205 derivative plasmids disrupted in loci "a", "b", "c", "d", and "e" (lanes 3-7).

B) Extracts were of *E. coli* cells harboring: recombinant plasmid pILL753 containing the *H. pylori ure A* and *ure B* genes (Labigne et al., 1991) (lane 1); and pILL205 derivative plasmids disrupted in loci "f", "g", "h", and "i" (lanes 2-5). The small arrow heads indicate polypeptides of approximately 30 and 66 kilodaltons, which represent putative *UreA* and *UreB* gene products of *H. felis*. The large arrow heads in panel B indicate the corresponding gene products of *H. pylori*, which cross-reacted with the anti-*H. felis* serum. The numbers indicate the molecular weights (in thousands) of the protein standards.

Fig. 3. Nucleotide sequence of the *H. felis* structural urease genes. Numbers above the sequence indicate the nucleotide positions as well as the amino acid position in each of the two *UreA* and *UreB* polypeptides. Predicted amino acid sequences for *UreA* (bp 43 to 753) and *UreB* (766 to 2616) are shown below the sequence. The putative ribosome-binding site (Shine-Dalgarno sequence, SD) is underlined.

Fig. 4. Comparison of sequences for the structural urease genes of *H. felis* to:

- a) the sequence of the two subunits of *H. pylori* urease (Labigne et al., 1991);
- b) the sequence of the three subunits of *Proteus mirabilis* urease (Jones and Mobley, 1989);

c) the sequence of the single subunit of jack bean urease. Margin gaps (shown by dashes) have been introduced to ensure the best alignment. *, amino acids identical to those of the *H. felis* sequence; =, amino acids shared by ureases; , amino acids unique to the *Helicobacter* species. The percentages refer to the number of amino acids that are identical to those of the *H. felis* urease. Abbreviations: *H.f.*, *Helicobacter felis*; *H.p.*, *Helicobacter pylori*; *P.m.*, *Proteus mirabilis*; J.B., jack bean.

Fig. 5. Restriction map of the recombinant plasmids pILL689, pILL685, and pILL691. The construction of these plasmids is described in detail in Table 5. Km within triangles depicts the site of insertion of the kanamycin cassette, which led to the construction of plasmids pILL687, pILL688, and pILL696 (Table 5). Boxes underneath the maps indicate the position of the three genetic elements deduced from the nucleotide sequence, namely IS5, *hsp A* and *hsp B*.

Fig. 6. Nucleotide sequence of the *Helicobacter pylori* heat shock protein gene cluster. The first number above the sequence indicates the nucleotide positions, whereas the second one numbers the amino acid residue position for each of the HspA and HspB protein. The putative ribosome-binding sequences (Shine-Dalgarno [SD] sites) are underlined.

Fig. 7. Comparison of the deduced amino-acid sequence of *Helicobacter pylori* HspA (A) or HspB (B) with that of other GroEL-like (A) or GroES-like (B) proteins. Asterisks mark amino acids identical with those in the *Helicobacter pylori* HspA or HspB sequences.

Fig. 8. Expression of the *Helicobacter pylori* HspA heat shock proteins in *E. coli* minicells. The protein bands with apparent molecular masses of 58 and 13 kDA, corresponding to the *Helicobacter pylori* HspA and HspB heat shock proteins are clearly visible in the lanes corresponding to plasmids pILL689 and

pILL692 and absent in the vector controls (pILL570 and pACYC177, respectively).

Fig. 9. Nucleotide sequence of the *Helicobacter felis* Ure I gene and deduced amino acid sequence.

Fig. 10. Comparison of the amino acid sequence of the Ure I proteins deduced from the nucleotide sequence of the Ure I gene of *Helicobacter felis* and that of *Helicobacter pylori*.

Fig. 11. Genetic code. Chain-terminating, or "nonsense", codons. Also used to specify the initiator formyl-Met-tRNA^{Met}_F. The Val triplet GUG is therefore "ambiguous" in that it codes both valine and methionine.

Fig. 12. Signification of the one-letter and three-letter amino-acid abbreviations.

Fig. 13. Purification of *H. pylori* UreA-MBP recombinant protein using the pMAL expression vector system. Extracts from the various stages of protein purification were migrated on a 10% resolving SDS-polyacrylamide gel. Following electrophoresis, the gel was stained with Coomassie blue. The extracts were: 1) non-induced cells; 2) IPTG-induced cells; French press lysate of induced cell extract; 5) eluate from amylose resin column; 6) eluate from anion exchange column (first passage); 7) eluate from anion exchange column (second passage); and 8) SDS-PAGE standard marker proteins.

Fig. 14. Recognition of UreA recombinant fusion proteins by polyclonal rabbit anti-*Helicobacter* sera. Protein extracts of maltose-binding protein (MBP, lane 1), *H. felis* UreA-MBP (lane 2), and *H. pylori* UreA-MBP (lane 3) were Western blotted using rabbit polyclonal antisera (diluted 1:5000) raised against whole cell extracts of *H. pylori* and *H. felis*. The purified fusion proteins are indicated by an arrow. Putative degradation products of the proteins are shown by an asterisk.

Fig. 15. Recognition of UreB recombinant fusion proteins by rabbit antisera raised against purified homologous and heterologous UreB proteins. Nitrocellulose membranes were blotted with the following extracts: 1) standard protein markers; 2) *H. felis* UreA-MBP; 3) MBP; 4) *H. pylori* UreA-MBP. The membranes were reacted with polyclonal rabbit antisera (diluted 1:5000) raised against MBP-fused *H. pylori* and *H. felis* UreB sub-units, respectively. The molecular weights of standard proteins are presented on the left-hand side of the blots.

Fig. 16. Western blot analysis of *H. pylori* and *H. felis* whole cell extracts with antisera raised against purified UreB MBP-fused recombinant proteins. SDS-PAGE whole extracts of *H. Felis* (lane 1) and *H. pylori* (lane 2) cells were reacted with polyclonal rabbit antisera raised against purified *H. pylori* UreB and *H. felis* UreB MBP-fused proteins (sera diluted 1:5000). The difference in gel mobility of the respective non-recombinant UreB sub-units of *H. felis* and *H. pylori* can be seen. The numbers on the left refer to the molecular weights of standard marker proteins.

Fig. 17. SDS-PAGE analysis of material eluted from the amylose column (lanes 2 and 3) or from the Ni-NTA column following elution: with buffer E (pH 4.5), lanes 4 and 5; or buffer C (pH 6.3), lanes 6 and 7. Material eluted from a lysate of MC1061 (PILL933) (lanes 2, 3, 5, and 7) and material eluted from a lysate of MC1061 (PMAL-c2) (lanes 4 and 6). Lane 3 contains the same material as in lane 2 except that it was resuspended in buffer E, thus demonstrating that buffer E is responsible for dimer formation of the MBP-HspA subunit, as seen in lanes 3 and 5.

Fig. 18. Serum IgG responses to MBP (bottom), MBP-HspA (top) and MBP-HspB (middle) of 28 *H. pylori* infected patients (squares, left) and 12 uninfected patients (circles, right). The optical density of each serum in the ELISA assay described in Experimental Procedures was read at 492 nm, after a

30 mn incubation. The sizes of the symbols are proportional to the number of sera giving the same optical density value.

Fig. 19. Measurement by ELISA of serum antibodies (IgG₁ and IgG_{2a} isotypes) in mice immunized with recombinant *H. pylori* antigens. A₄₉₂ values for individual serum samples (diluted 1:100) are presented. Horizontal lines represent the mean A₄₉₂ values for each set of data.

Fig. 20. Immunoblot analyses of total cell extracts of *H. felis* (lane 1) and *H. pylori* (lane 2) using rabbit antisera raised against recombinant *H. pylori* HspA and HspB antigens (dilution 1:5000). Arrows refer to cross-reactive proteins: (i) monomeric and (ii) dimeric forms of HspA antibody-reactive proteins are indicated. Protein standards are indicated on the right-hand side of each of the blots (numbers are in kDa). Immunoreactants on the anti-HspA blotted membrane were revealed directly with a peroxidase-labelled secondary antibody, whilst antigens on the anti-HspB were detected using a biotinylated secondary antibody/streptavidin-peroxidase procedure. The latter was found to give higher background staining and when used to detect immunoreactants on membranes blotted with the anti-HspA antibody, produced very weak signals.

Best Mode for Carrying Out the Invention

An immunogenic composition capable of inducing antibodies against *Helicobacter* infection can be characterized in that it comprises:

- i) at least one sub-unit of a urease structural polypeptide from *Helicobacter pylori*, or a fragment thereof, defined by two restriction sites or comprised between 6 to 100 amino acids or delineated by two specific oligonucleotides targeting any sequence of 300 bp, said fragment being recognized by antibodies reacting with *Helicobacter felis* urease, and/or at least one sub-unit of a urease structural polypeptide from *Helicobacter felis*, or a fragment thereof, said fragment being recognized by antibodies reacting with *Helicobacter pylori* urease;

ii) and/or a Heat Shock protein (Hsp), or chaperonin, from *Helicobacter*, or a fragment of said protein.

Preferably, the immunogenic composition is capable of inducing protective antibodies.

According to a preferred embodiment, the immunogenic composition of the invention contains, as the major active ingredient, at least one sub-unit of a urease structural polypeptide from *Helicobacter pylori* and/or *Helicobacter felis*. The expression "urease structural polypeptide" signifies, in the context of the present invention, the enzyme of *Helicobacter pylori* or *Helicobacter felis*, probably a major surface antigen composed of two repeating monomeric sub-units, a major sub-unit (product of the *UreB* gene) and a minor sub-unit product of the *UreA* gene, and which, when complemented by the presence of the products of the accessory genes of the urease gene cluster, are responsible for urease activity i.e., the hydrolysis of urea to liberate NH_4^+ in the two *Helicobacter* species. It is to be understood that in the absence of the accessory gene products, the urease structural polypeptides do not exhibit enzymatic activity, but are recognized by antibodies reacting with *H. felis* or *H. pylori* urease.

The term "immunogenic composition" signifies, in the context of the invention, a composition comprising a major active ingredient as defined above, together with any necessary ingredients to ensure or to optimize an immunogenic response, for example adjuvants, such as mucosal adjuvant, etc.

The *Helicobacter pylori* urease structural polypeptide has been described and sequenced by Labigne et al., 1991. The polypeptide described in this paper is particularly appropriate for use in the composition of the present invention. However, variants showing functional homology with this published sequence may be used, which comprise amino acid substitutions, deletions or insertions provided that the immunological characteristics of the polypeptide insofar as its cross-reactivity with anti-*Helicobacter felis* urease antibodies is concerned, are

maintained. Generally speaking, the polypeptide variant will show a homology of at least 75% and preferably about 90% with the included sequence.

A fragment of the *Helicobacter pylori* urease structural polypeptide may also be used in the immunogenic composition of the invention, or at least one sub-unit of a urease structural polypeptide from *Helicobacter pylori*, or a fragment thereof, defined by two restriction sites or comprised between 6 to 100 amino acids or delineated by two specific oligonucleotides targeting any sequence of 300 bp, provided that the fragments are recognized by antibodies reacting with *helicobacter felis* urease. Such a fragment will generally be comprised of at least 6 amino acids, for example, from 6 to 100 amino acids, preferably about 20-25. Advantageously, the fragment carries epitopes unique to *Helicobacter*.

Nucleic acid and amino-acid sequences may be interpreted in the context of the present invention by reference to Figs. 11 and 12, showing the genetic code and amino acid abbreviations respectively.

The *Helicobacter felis* urease structural polypeptide suitable for use in the present invention is preferably that encoded by part of the plasmid pILL205 (deposited at the CNCM on 25th August 1993, under number: CNCM I-1355), and whose amino acid sequence is shown in Fig. 3 (subunits A and B). Again, a variant of this polypeptide comprising amino acid substitutions, deletions or insertions with respect to the Fig. 3 sequence may be used provided that the immunological cross-relationship with *Helicobacter pylori* urease is maintained. Such a variant normally exhibits at least 90% homology or identity with the Fig. 3 sequence. An example of such variants are the urease A and B sub-units from *Helicobacter heilmannii* (Solnick et al., 1994), shown to have 80% and 92% identity with the *H. felis* urease A and B sub-units, respectively.

Fragments of this urease or variants may be used in the immunogenic composition provided that the fragments are recognized by antibodies reacting with *Helicobacter pylori* urease. Again, the length of such a fragment is usually

at least 6 amino acids, for example, from 6 to 100, preferably about 20 to 25. Preferably, the fragment carries epitopes unique to *Helicobacter*.

If variants or fragments of the native urease sequences are employed in the immunogenic composition of the invention, their cross-reactivity with antibodies reacting with urease from the other *Helicobacter* species can be tested by contacting the fragment or the variant with antibodies, preferably polyclonal raised to either the native or the recombinant urease or, alternatively, to whole *Helicobacter*. Preferably, the variants and fragments give rise to antibodies which are also capable of reacting with *H. heilmannii* urease. Cross protection to infection by *H. heilmannii* is therefore also obtained by the immunogenic composition of the invention.

The use of fragments of the urease structural genes is particularly preferred since the immunological properties of the whole polypeptide may be conserved whilst minimizing risk of toxicity.

The active component of the immunogenic composition of the invention may be comprised of one sub-unit only of the urease structural polypeptide, that is either sub-unit A or sub-unit B products of the *UreA* and *UreB* genes, respectively. Compositions comprising only the urease sub-unit UreB, of either *H. pylori* or *H. felis*, or variants and fragments as defined above, are particularly advantageous. Most preferred are homologous systems wherein the urease sub-unit, particularly sub-unit B, is derived from the organism against which protection is sought, e.g., *H. felis* sub-unit B against *H. felis* infection. However, the composition may contain both A and B sub-units, which are normally present as distinct polypeptides. However, it is possible, when the polypeptide is produced by recombinant means, to use a fusion protein comprising the entire sequences of the A and B gene products by the suppression of the stop-codon separating the two adjacent coding sequences.

The urease component of the immunogenic composition, whether sub-unit A or sub-unit B, may be used in the form of translational fusion proteins, for example with the Maltose-Binding-Protein (MBP). Other suitable fusions are exemplified in International Patent Application WO 90/11360. Another example of a suitable fusion protein is the "QIAexpress" system commercialized by QIAGEN, USA, which allows the 6xHis tag sequence to be placed at the 5' or 3' end of the protein coding sequence. The use of the active ingredients in the form of fusion proteins is, however, entirely optional.

According to a further preferred embodiment, the immunogenic composition of the invention may comprise in addition to or instead of the urease structural polypeptide defined above, a Heat Shock Protein also known as a "chaperonin" from *Helicobacter*. These chaperonins have been elucidated by the inventors in the context of the present invention. Preferably, the chaperonin is from *Helicobacter pylori*. Such an Hsp may be the urease-associated HspA or HspB or a mixture of the two, having the amino acid sequence illustrated in Fig. 6. These polypeptides are encoded by the plasmid pILL689 (deposited at CNCM on 25th August 1993, under number: CNCM I-1356). Particularly preferred is the *H. pylori* HspA protein, either alone or in combination with HspB.

It is also possible to use, as Hsp component, according to the invention, a polypeptide variant in which amino acids of the Fig. 6 sequence have been replaced, inserted or deleted, the said variant normally exhibiting at least 75%, and preferably at least 85% homology with the native Hsp. The variants preferably exhibit at least 75%, for example at least 85% identity with the native Hsp.

The variants may further exhibit functional homology with the native polypeptide. In the case of the Hsp components, "functional homology" means the capacity to enhance urease activity in a microorganism capable of expressing active urease, and/or the capacity to block infection by *Helicobacter*, particularly

H. felis and *H. pylori*. The property of enhancing urease activity may be tested using the quantitative urease activity assay described below in the examples. Fragments of either or both of the HspA and HspB polypeptides, preferably having at least 6 amino acids, may be used in the composition. The fragments or variants of the Hsp component used in the immunogenic composition of the invention are preferably capable of generating antibodies, which block the infection against *H. pylori* or *H. felix*. The presence of the chaperonins in the composition enhances the protection against *Helicobacter pylori* and *felis*.

The Hsp component of the immunogenic composition, whether HspA or HspB, can be used in the form of a translational fusion protein, for example with the Maltose-Binding-Protein (MBP). As for the urease component, other suitable fusion partners are described in International Patent Application WO 90/11360. The "QIAexpress" system of QIAGEN, USA, may also be used. Again, the use of the proteins in the form of fusion proteins is entirely optional.

According to the invention, therefore, the immunogenic composition may comprise either a urease structural polypeptide as defined above, or a *Helicobacter* Hsp, particularly HspA or a combination of these immunogens.

According to a preferred embodiment, the immunogenic composition comprises, as urease component or a fragment thereof, both the A and/or B sub-units or fragments of urease of *Helicobacter felis* (i.e., without *H. pylori* urease) the urease component can be associated or not to the Hsp A and/or Hsp B of *Helicobacter pylori*. Alternatively, the A and B sub-units of the *Helicobacter felis* urease may be used together with those of *H. pylori*, but without chaperonin component.

The immunological cross-reactivity between the ureases of the two different *Helicobacter* species enables the use of one urease only in the composition, preferably that of *Helicobacter felis*. The protective antibodies induced by the common epitopes will, however, be active against both

Helicobacter pylori and *Helicobacter felis*. It is also possible that the composition induce protective antibodies to other species of *Helicobacter* if the urease polypeptide or fragment carries epitopes occurring also on those other species.

In a preferred embodiment, the composition of the invention comprises a mixture of antigens of Helicobacter wherein said mixture consists essentially of UreB and HspA of H. pylori or polypeptides having at least 75 % and preferably 80 to 90 % similarity with said UreB or HspA, or fragments thereof capable of eliciting antibodies recognized by H. pylori or an immune cellular response against H. pylori infection.

In another specific embodiment of the invention, the composition comprises a mixture consisting essentially of ureB and HspA of H. felis or polypeptides having at least 75 % and preferably 80 to 90 % similarity with said UreB or HspA, or fragments thereof capable of inducing antibodies recognized by H. pylori and/or by H. felis or an immune cellular response against an H. pylori and/or H. felis infection.

The preparation of such antibodies is described for illustration in the examples.

The invention further relates to fragments having the above properties, which fragments comprise between 9 and 200 aminoacid residues. These fragments can be used for inducing antibodies. Such fragments can be prepared by usual synthesis for example as proposed by « Applied Biosystem »

As a preferred fragment for the preparation of the invention, the C-terminal sequence of HspA of H. pylori replying to the following amino-acid sequence is used:

G S C C H T G N H D H K H A K E H E A C C H D H K K H.

The composition of the invention preferably contains an amount of antigens or fragments thereof sufficient to elicit an immune response in a host to whom it is administered.

The response can be a cellular and/or a humoral immune response.

Advantageously the antigen or fragments thereof are recombinant antigens expressed in a purified form or included in fusion proteins. Some examples of antigens or gene products and proteinaceous material suitable for the formation of fusion proteins are cited in the present description and should be regarded as appropriate for preparing fusion proteins with UreB or HspA antigens or fragments thereof.

In a particular embodiment, the composition described hereabove is substantially free of UreA antigen and in another embodiment, is substantially free of other H. pylori or H. felis antigens.

Further components can be added to the composition, including adjuvants and for instance a mucosal adjuvant, for example, cholera or E. coli holotoxins.

The composition described hereabove can be formulated as a pharmaceutical composition; in such a case physiologically acceptable excipients may be added.

The composition of the invention is advantageously used as an immunogenic composition or a vaccine, together with physiologically acceptable excipients and carriers and, optionally, with adjuvants, haptens, carriers, stabilizers, etc. Suitable adjuvants include muramyl dipeptide (MDP), complete and incomplete Freund's adjuvants (CFA and IFA) and alum. The vaccine compositions are normally formulated for oral administration.

The vaccines are preferably for use in man, but may also be administered in non-human animals, for example for veterinary purposes, or for use in animals such as mice, cats and dogs.

They are in particular suitable for protecting against Helicobacter, especially H. pylori.

The immunogenic compositions administered by suitable routes into animals raises an immune response and especially raises the synthesis *in vivo* of specific antibodies, which can be used for therapeutic or curative purposes, for example in passive immunity.

Especially a composition comprising a mixture of UreB and HspA of H. pylori and/or H. felis or related polypeptides or fragments thereof is interesting for inducing or enhancing a protective response against mucosal infection by Helicobacter pylori in a host to whom it is administered.

The invention also relates to the proteinaceous materials used in the immunogenic composition and to proteinaceous material encoded by the urease gene clusters other than the A and B urease structural sub-units. "Proteinaceous material" means any molecule comprised of chains of amino acids, e.g., peptides, polypeptides or proteins, fusion or mixed proteins (i.e. an association of 2 or more proteinaceous materials, all or some of which may have immunogenic or immunomodulation properties), either purified or in a mixture with other proteinaceous or non-proteinaceous material. "Polypeptide" signifies a chain of amino acids whatever its length and englobes the term "peptide". The term "fragment" means any amino acid sequence shorter by at least one amino acid than the parent sequence and comprising a length of amino acids, e.g., at least 6 residues, consecutive in the parent sequence.

The peptide sequences of the invention, may for example, be obtained by chemical synthesis, using a technique such as the Merrifield technique and synthesizer of the type commercialized by Applied Biosystems.

In particular, the invention relates to proteinaceous material characterized in that it comprises at least one of the *Helicobacter felis* polypeptides encoded by the urease gene cluster of the plasmid pILL205 (CNCM I-1355), including the

structural and accessory urease polypeptides, or a polypeptide having at least 90% homology with said polypeptides, or a fragment thereof. Of particular interest are the gene products of the *ure A* and *ure B* genes, as illustrated in Fig. 3, or a variant thereof having at least 90% homology or a fragment having at least 6 amino acids. The fragments and the variants are recognized by antibodies reacting with *Helicobacter pylori* urease.

Another polypeptides encoded by the accessory genes of the urease gene cluster is the gene product of *Ure I*, as illustrated in Fig. 9, which also forms part of the invention. Also included is a variant of the *Ure I* product having at least 75% homology, preferably at least 85%, or a fragment of the gene product or of the variant having at least 6 amino acids. The variant preferably has the capacity to modulate the expression of urease activity. The urease activity can be detected by using the following test: 10^9 bacteria containing the *Ure I* gene product variant are suspended in 1 ml of urea-indole medium and incubated at 37° C. The hydrolysis of the urea leads to the release of ammonium, which increases pH and induces a color change from orange to fuschia-red.

It is also possible that a fragment of the *Ure I* gene product, if it has a length of, for example, at least 70 or 100 amino acids, may also exhibit this functional homology with the entire polypeptide.

The fragments of *Ure I* polypeptide or of the variant preferably are capable of inducing the formation of antibodies which interfere with the activation process of the urease apoenzyme.

The invention also relates to the proteinaceous material comprising at least one of the heat shock proteins or chaperonins of *Helicobacter pylori* or a fragment thereof. Particularly preferred are the HspA and HspB polypeptides as illustrated in Fig. 6 or a polypeptide having at least 75%, and preferably at least 80 or 90%, homology or identity with the said polypeptide. A particularly

preferred fragment of the *Helicobacter pylori* HspA polypeptide is the C-terminal sequence:

G S C C H T G N H D H K H A K E H E A C C H D H K K H

or a sub-fragment of this sequence having at least 6 consecutive amino acids. This C-terminal sequence is thought to act as a metal binding domain allowing binding of, for example, nickel or divalent cations.

E. coli strains containing various subsets of the *H. pylori* urease subunits:

- *E. coli* MC1061 (pILL918) [CNCM registration number I-1336] expressing a UreA peptide (AA N°19 to AA N°238) fused to MalE
- *E. coli* MC1061 (pILL923) [CNCM registration number I-1338] expressing a UreA peptide (AA N°58 to AA N°238) fused to MalE
- *E. coli* MC1061 (pILL924) [CNCM registration number I-1339] expressing a UreA peptide (AA N°184 to AA N°238) fused to MalE
- *E. coli* MC1061 (pILL928) [CNCM registration number I-1341] expressing a UreA peptide (AA N°205 to AA N°569) fused to MalE
- *E. coli* MC1061 (pILL931) [CNCM registration number I-1342] expressing a UreA peptide (AA N°400 to AA N°569) fused to MalE

HspA and HspB of *H. felis* are detected as shown in Fig. 20. Antibodies raised against recognized MBP HspA or MBP HspB of *H. pylori* recognized HspA where HspB of *H. felis*.

The proteinaceous material of the invention may also comprise or consist of a fusion or mixed protein including at least one of the sub-units of the urease structural polypeptide of *H. pylori* and/or of *H. felis*, or fragments or variants thereof as defined above. Particularly preferred fusion proteins are the Mal-E fusion proteins and QIAexpress system fusion proteins (QIAGEN, USA) as detailed above. The fusion or mixed protein may include, either instead of or in

addition to the urease sub-unit, a Heat Shock Protein, or fragment or variant thereof, as defined above.

The invention also relates to monoclonal or polyclonal antibodies to the protein materials described above. More particularly, the invention relates to antibodies or fragments thereof to any one of the *Helicobacter felis* polypeptides encoded by the urease gene cluster of the plasmid pILL205 (CNCM I-1355), including the structural and accessory urease polypeptides, that is, structural genes *UreA* and *UreB* and the accessory genes known as *Ure E*, *Ure F*, *Ure G*, *Ure H* and *Ure I*. The antibodies may also be directed to a polypeptide having at least 90% homology with any of the above urease polypeptides or to a fragment thereof preferably having at least 6 amino acids. The antibodies of the invention may specifically recognize *Helicobacter felis* polypeptides expressed by the urease gene cluster. In this case, the epitopes recognized by the antibodies are unique to *Helicobacter felis*. Alternatively, the antibodies may include or consist of antibodies directed to epitopes common to *Helicobacter felis* urease polypeptides and to *Helicobacter pylori* urease polypeptides. If the antibodies recognize the accessory gene products, it is particularly advantageous that they cross-react with the *Helicobacter pylori* accessory gene product. In this way, the antibodies may be used in therapeutic treatment of *Helicobacter pylori* infection in man by blocking the urease maturation process.

Particularly preferred antibodies of the invention recognize the *Helicobacter felis* *UreA* and/or *UreB* gene products, that is the A and B urease sub-units. Advantageously, these antibodies also cross-react with the *Helicobacter pylori* A and B urease sub-units, but do not cross-react with other ureolytic bacteria. Such antibodies may be prepared against epitopes unique to *Helicobacter* (see Fig. 4), or alternatively, against the whole polypeptides followed by screening out of any antibodies reacting with other ureolytic bacteria.

The invention also concerns monoclonal or polyclonal antibodies to the Hsps or fragments thereof, particularly to the HspA and/or HspB protein illustrated in Figure 6. Polypeptides having at least 75%, and preferably at least 80%, or 90%, homology with the Hsps may also be used to induce antibody formation. These antibodies may be specific for the *Helicobacter pylori* or *H. felis* chaperonins or, alternatively, they may cross-react with GroEL-like proteins or GroES-like proteins from bacteria other than *Helicobacter*, depending upon the epitopes recognized. Fig. 7 shows the homologous regions of HspA and HspB with GroES-like proteins and GroEL-like proteins, respectively, from various bacteria. Particularly preferred antibodies are those specific for either the HspA or HspB chaperonins or those specifically recognizing the HspA C-terminal sequence having the metal binding function. Again, use of specific fragments for the induction of the antibodies ensures production of *Helicobacter*-specific antibodies.

The invention further relates to antibodies obtained against a composition comprising a mixture of UreB and HspA antigens of *H. pylori*, or a mixture of polypeptides having at least 75 %, preferably 80 or 90 % similarity with said UreB or HspA antigens, or fragments thereof.

These antibodies can be directed against one of the above antigens, or can be a mixture of antibodies against these different antigens of the composition.

Furthermore these antibodies are specific to *H. pylori* or to *H. felis* or to the contrary cross react with both strains.

The antibodies of the invention may be prepared using classical techniques. For example, monoclonal antibodies may be produced by the hybridoma technique, or by known techniques for the preparation of human antibodies, or by the technique described by Marks et al. (Journal of Molecular Biology, 1991, 222, p. 581-597).

The invention also includes fragments of any of the above antibodies produced by enzyme digestion. Of particular interest are the Fab and F(ab')₂ fragments. Also of interest are the Facb fragments.

The invention also relates to purified antibodies or serum obtained by immunization of an animal, e.g., a mammal, with the immunogenic composition, the proteinaceous material or fragment, or the fusion or mixed protein(s) of the invention, followed by purification of the antibodies or serum. Such protein can be the product of one of the genes of urease cluster either *H. pylori* or *H. felis* associated or not with the product of *HspA* or *HspB* of *H. pylori* or *H. felis* genes.

Also concerned is a reagent for the *in vitro* detection of *H. pylori* infection containing at least these antibodies or serum, optionally with reagents for labelling the antibodies, e.g., anti-antibodies etc.

The invention further relates to nucleic acid sequences coding for any of the above proteinaceous materials including peptides. In particular, the invention relates to a nucleic acid sequence characterized in that it comprises:

- i) a sequence coding for the *Helicobacter felis* and/or *H. pylori* urease and/or accessory polypeptides as defined above, and/or a sequence coding for the Hsp of *H. pylori* or *H. felis* as defined above; or
- ii) a sequence complementary to sequence (i); or
- iii) a sequence capable of hybridizing to sequence (i) or (ii) under stringent conditions; or
- iv) a fragment of any of sequences (i), (ii) or (iii) comprising at least 10 nucleotides.

Preferred nucleic acid sequences are those comprising all or part of the sequence of plasmid pILL205 (CNCM I-1355), for example the sequence of Fig. 3, in particular that coding for the gene product of *UreA* and for *UreB* or the sequence of Fig. 9 (*UreI*), or a sequence capable of hybridizing with these sequences under stringent conditions, or a sequence complementary to these

sequences, or a fragment comprising at least 10 consecutive nucleotides of these sequences.

Other preferred sequences are those comprising all or part of the sequence of plasmid pILL689 (CNCM I-1356), for example the sequence of Fig. 6, in particular that coding for *HspA* and/or *HspB*, or a sequence complementary to this sequence, or a sequence capable of hybridizing to this sequence under stringent conditions, or a fragment thereof.

High stringency hybridization conditions in the context of the invention are the following:

- 5 x SSC;
- 50% formamide at 37°C;

or:

- 6 x SSC;
- Denhard medium at 68°C.

The sequences of the invention also include those hybridizing to any of sequences (i), (ii) and (iii) defined above under non-stringent conditions, that is:

- 5 x SSC;
- 0.1% SDS;
- 30 or 40% formamide at 42°C, preferably 30%.

The term "complementary sequences" in the context of the invention signifies "complementary" and "reverse" or "inverse" sequences.

The nucleic acid sequences may be DNA or RNA.

The sequences of the invention may be used as nucleotide probes in association with appropriate labelling means. Such means include radioactive isotopes, enzymes, chemical or chemico-luminescent markers, fluorochromes, haptens, or antibodies. The markers may optionally be fixed to a solid support, for example a membrane or particles.

As a preferred marker, radioactive phosphorous (^{32}P) is incorporated at the 5'-end of the probe sequence. The probes of the invention comprise any fragment of the described nucleic acid sequences and may have a length for example of at least 45 nucleotides, for example 60, 80 or 100 nucleotides or more. Preferred probes are those derived from the *UreA*, *UreB*, *Ure I*, *HspA* and *HspB* genes.

The probes of the invention may be used in the *in vitro* detection of *Helicobacter* infection in a biological sample, optionally after a gene amplification reaction. Most advantageously, the probes are used to detect *Helicobacter felis* or *Helicobacter pylori*, or both, depending on whether the sequence chosen as the probe is specific to one or the other, or whether it can hybridize to both. Generally, the hybridization conditions are stringent in carrying out such a detection.

The invention also relates to a kit for the *in vitro* detection of *Helicobacter* infection, characterized in that it comprises:

- a nucleotide probe according to the invention, as defined above;
- an appropriate medium for carrying out a hybridization reaction between the nucleic acid of *Helicobacter* and the probe; and
- reagents for the detection of any hybrids formed.

The nucleotide sequences of the invention may also serve as primers in a nucleic acid amplification reaction. The primers normally comprise at least 10 consecutive nucleotides of the sequences described above and preferably at least 18. Typical lengths are from 25 to 30 and may be as high as 100 or more consecutive nucleotides. Such primers are used in pairs and are chosen to hybridize with the 5'- and 3'-ends of the fragment to be amplified. Such an amplification reaction may be performed using for example the PCR technique (European patent applications EP200363, 201184 and 229701). The Q-^ab-

replicase technique (Biotechnology, vol. 6, Oct. 1988) may also be used in the amplification reaction.

The invention also relates to expression vectors characterized in that they contain any of the nucleic acid sequences of the invention. Particularly preferred expression vectors are plasmids pILL689 and pILL205 (CNCM I-1356 and CNCM I-1355, respectively). The expression vectors will normally contain suitable promoters, terminators and marker genes, and any other regulatory signals necessary for efficient expression.

The invention further relates to prokaryotic or eukaryotic host cells stably transformed by the nucleic acid sequences of the invention. As examples of hosts, mention may be made of higher eukaryotes such as CHO cells and cell-lines; yeast; prokaryotes including bacteria such as *E. coli*, e.g. *E. coli* HB 101, *Shigellae* or *Salmonella*, *Mycobacterium tuberculosis*, viruses including baculovirus and vaccinia. Usually the host cells will be transformed by vectors. However, it is also possible within the context of the invention to insert the nucleic acid sequences by homologous recombination, using conventional techniques. For example, WO 90.11354 (Brulet et al.) describes the technology to carry out an homologous recombination in eukaryotic cells.

By culturing the stably transformed hosts of the invention, the *Helicobacter* urease polypeptide material and, where applicable, the Hsp material can be produced by recombinant means. The recombinant proteinaceous materials are then collected and purified. Pharmaceutical compositions are prepared by combining the recombinant materials with suitable excipients, adjuvants, and optionally, any other additives, such as stabilizers.

The invention also relates to plasmids pILL920 (deposited at CNCM on 20.07.1993, under accession number I-1337) and pILL927 (CNCM I-1340, deposited on 20.07.1993) constructed as described in the examples below.

The invention covers also the DNA (or RNA derived from such DNA) purified from the expression vectors and used as immunogen capable of inducing an immune response in a host (cellular or antibody response).

Examples

I. Cloning, Expression And Sequencing Of *H. felis* Urease Gene:

A. Experimental Procedures For Part I:

1. Bacterial strains and culture conditions:

H. felis (ATCC 49179) was grown on blood agar base no. 2 (Oxoid) supplemented with 5% (v/v) lysed horse blood (BioMerieux) and an antibiotic supplement consisting of 10 ng ml⁻¹ vancomycin (Lederle Laboratories), 2.5 µg ml⁻¹ polymyxin B (Pfizer), 5 µg ml⁻¹ trimethoprim (Sigma Chemical Co.) and 2.5 µg ml⁻¹ amphotericin B (E.R Squibb and Sons, Inc.). Bacteria were cultured on freshly prepared agar plates and incubated, lid uppermost, under microaerobic conditions at 37°C for 2-3 days. *E. coli* strains HB101 (Boyer and Roulland-Dussoix, 1969) and MC1061 (Maniatis et al., 1983), used in the cloning experiments, were grown routinely in Luria broth without glucose added or on Luria agar medium, at 37°C. Bacteria grown under nitrogen-limiting conditions were passaged on a nitrogen-limiting solid medium consisting of ammonium-free M9 minimal medium (pH 7.4) supplemented with 0.4% (w/v) D-glucose and 10 mM L-arginine (Cussac et al., 1992).

2. DNA manipulations:

All standard DNA manipulations and analyses, unless mentioned otherwise, were performed according to the procedures described by Maniatis et al. (1983).

3. Isolation of *H. felis* DNA:

Total genomic DNA was extracted by an sarkosyl-proteinase K lysis procedure (Labigne-Roussel et al., 1988). Twelve blood agar plates inoculated with *H. felis* were incubated in an anaerobic jar (BBL) with an anaerobic gaspak (BBL 70304) without catalyst, for 1-2 days at 37°C. The plates were harvested in 50 ml of a 15% (v/v) glycerol - 9% (w/v) sucrose solution and centrifuged at 5,000

rpm (in a Sorvall centrifuge), for 30 min at 4°C. The pellet was resuspended in 0.2 ml 50 mM D-glucose in 25 mM Tris-10 mM EDTA (pH 8.0) containing 5 mg ml⁻¹ lysozyme and transferred to a VTI65 polyallomer quick seal tube. A 0.2 ml aliquot of 20 mg ml⁻¹ proteinase K and 0.02 ml of 5M sodium perchlorate were added to the suspension. Cells were lysed by adding 0.65 ml of 0.5M EDTA - 10% (w/v) and incubated at 65°C until the suspension cleared (approximately 1 h). The volume of the tube was completed with a CsCl solution consisting of 100 ml of 126 g CsCl, 1 ml aprotinine, 99 ml TES buffer (30 mM Tris, 5 mM EDTA, 50 mM NaCl (pH 7.5). Lysates were centrifuged at 45,000 rpm, for 18°C. Total DNA was collected and dialyzed against TE buffer (10 mM Tris, 1 mM EDTA), at 4°C.

4. Cosmid cloning:

Chromosomal DNA from *H. felis* was cloned into cosmid vector pILL575, as previously described (Labigne et al., 1991). Briefly, DNA fragments arising from a partial digestion with *Sau3A* were sized on a (10 to 40%) sucrose density gradient and then ligated into a *Bam*HI-digested and dephosphorylated pILL575 DNA preparation. Cosmids were packaged into phage lambda particles (Amersham, In Vitro packaging kit) and used to infect *E. coli* HB101. To screen for urease expression, kanamycin-resistant transductants were replica-plated onto solid nitrogen-mimicking medium (see above) containing (20 µg ml⁻¹) kanamycin that had been dispensed into individual wells of microtitre plates (Becton Dickinson). The microtiter plates were incubated aerobically at 37°C for 2 days before adding 0.1 ml urease reagent (Hazell et al., 1987) to each of the wells. Ureolysis was detected within 5-6 h at 37°C by a color change in the reagent. Several urease-positive cosmid clones were restriction mapped and one was selected for subcloning.

5. Subcloning of *H. felis* DNA:

A large-scale CsCl plasmid preparation of cosmid DNA was partially digested *Sau*3A. DNA fragments (7 - 11 kb) were electroeluted from an agarose gel and purified using phenol-chloroform extractions. Following precipitation in cold ethanol, the fragments were ligated into Bg/III-digested plasmid pILL570 (Labigne et al., 1991) and the recombinant plasmids used to transform competent *E. coli* MC1061 cells. Spectinomycin-resistant transformants were selected and screened for urease expression under nitrogen-rich (Luria agar) and nitrogen-limiting conditions.

6. Quantitative urease activity:

Cultures grown aerobically for 2.5 days at 37°C were harvested and washed twice in 0.85% (w/v) NaCl. Pellets were resuspended in PEB buffer (0.1 M sodium phosphate buffer (pH 7.4) containing 0.01 M EDTA) and then sonicated by four 30-sec bursts using a Branson Sonifier Model 450 set at 30 W, 50% cycle. Cell debris was removed from the sonicates by centrifugation. Urease activities of the sonicates were measured in a 0.05 M urea solution prepared in PEB by a modification of the Berthelot reaction (Cussac et al., 1992). Urease activity was expressed as $\mu\text{mol urea min}^{-1}\text{mg}^{-1}$ bacterial protein.

7. Protein determination:

Protein concentrations were estimated with a commercial version of the Bradford assay (Sigma Chemicals).

8. Transposon mutagenesis:

Random insertional mutations were generated within cloned *H. felis* via a MiniTn3-Km delivery system (Labigne et al., 1992). In brief, *E. coli* HB101 cells containing the transposase-encoding plasmid pTCA were transformed with plasmid pILL570 containing cloned *H. felis* DNA. Transposition of the MiniTn3-Km element into the pILL570 derivative plasmids was effected via conjugation. The resulting cointegrates were then selected for resolved structures in the

presence of high concentrations of kanamycin (500 mg l⁻¹) and (300 mg l⁻¹).

9. SDS-PAGE and Western Blotting:

Solubilized cell extracts were analyzed on slab gels, comprising a 4.5% acrylamide stacking gel and 12.5% resolving gel, according to the procedure of Laemmli (Laemmli, 1970). Electrophoresis was performed at 200V on a mini-slab gel apparatus (Bio-Rad).

Proteins were transferred to nitrocellulose paper (Towbin et al., 1979) in a Mini Trans-Blot transfer cell (Bio-Rad) set at 100 V for 1 h (with cooling). Nitrocellulose membranes were blocked with 5% (w/v) purified casein (BDH) in phosphate-buffered saline (PBS) at room temperature, for 2 h (Ferrero et al., 1992). Membranes were reacted at 4°C overnight with antisera diluted in 1% (w/v) casein prepared in PBS. Immunoreactants were then detected using a biotinylated secondary antibody (Kirkegaard and Perry Lab.) in combination with avidin-peroxidase (KPL). A substrate solution composed of 0.3% (w/v) 4-chloro-1-naphthol (Bio-Rad) was used to visualize reaction products.

10. DNA Sequencing:

DNA fragments to be sequenced were cloned into M13mp18 and M13mp19 (Meissing and Vieira, 1982) bacteriophage vectors (Pharmacia). Competent *E. coli* JM101 cells were transfected with recombinant phage DNA and plated on media containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and isopropyl-β-D-thiogalactopyranoside. Plaques arising from bacteria infected with recombinant phage DNA were selected for the preparation of single-stranded DNA templates by polyethylene glycol treatment (Sanger et al., 1977). Single-stranded DNA sequenced according to the dideoxynucleotide chain termination method using a Sequenase kit (United States Biochemical Corp.).

11. Nucleotide sequence accession number:

The nucleotide accession number is X69080 (EMBL Data Library).

B. Results Of Part I Experiments:

1. Expression of urease activity by

H. felis cosmid clones:

Cloning of partially digested fragments (30 to 45 kb in size) of *H. felis* chromosomal DNA into the cosmid vector pILL575 resulted in the isolation of approximately 700 cosmid clones. The clones were subcultured on nitrogen-limiting medium in order to induce urease expression (Cussac et al., 1992). Six of these were identified as being urease-positive after 5-6 h incubation (as described in the Experimental procedures section). No other urease-positive cosmid clones were identified, even after a further overnight incubation. Restriction enzyme analysis of 3 clones harboring the urease-encoding cosmids revealed a common 28 kd DNA fragment. A cosmid (designated pILL199) containing DNA regions at both extremities of the common fragment was selected for subcloning.

2. Identification of *H. felis* genes required for urease expression when cloned in *E. coli* cells:

To define the minimum DNA region necessary for urease expression in *E. coli* cells, the urease-encoding cosmid pILL199 was partially digested with *Sau*3A and the fragments were subcloned into plasmid pILL570. The transformants were subcultured on nitrogen-rich and nitrogen-limiting media and screened for an urease-positive phenotype. Five transformants expressed urease activity when grown under nitrogen-limiting conditions, whereas no activity was detected following growth on nitrogen-rich medium. Restriction mapping analyses indicated that the urease-encoding plasmids contained inserts of between 7 and 11 kb. The plasmid designated pILL205 was chosen for further studies.

Random mutagenesis of cloned *H. felis* DNA was performed to investigate putative regions essential for urease expression in *E. coli* and to localize the region of cloned DNA that contained the structural urease genes. Random insertion mutants of prototype plasmid pILL205 were thus generated using the MiniTn3-Km element (Labigne et al., 1992). The site of insertion was restriction mapped for each of the mutated copies of pILL205 and cells harboring these plasmids were assessed qualitatively for urease activity (Fig. 1). A selection of *E. coli* HB101 cells harboring the mutated derivatives of pILL205 (designated "a" to "i") were then used both for quantitative urease activity determinations, as well as for the detection of the putative urease subunits by Western blotting.

The urease activity of *E. coli* HB101 cells harboring pILL205 was 1.2 ± 0.5 mmol urea $\text{min}^{-1}\text{mg}^{-1}$ bacterial protein (Table 1), which is approximately a fifth that of the parent *H. felis* strain used for the cloning. Insertion of the transposon at sites "a", "c", "d", "f" and "g" resulted in a negative phenotype, whilst mutations at sites "b", "e", "h" and "i" had no significant effect on the urease activities of clones harboring these mutated copies of pILL205 (Table 1). Thus mutagenesis of pILL205 with the MiniTn3-Km element identified three domains as being required for *H. felis* urease gene expression in *E. coli* cells.

3. Localization of the *H. felis* urease structural genes:

Western blot analysis of extracts of *E. coli* cells harboring pILL205 indicated the presence of two polypeptides of approximately 30 and 66 kDa, which cross-reacted with polyclonal *H. felis* rabbit antiserum (Fig. 2A). These proteins were not produced by bacteria carrying the vector (pILL570). Native *H. felis* urease has been reported to be composed of repeating monomeric subunits with calculated molecular weights of 30 and 69 kDa (Turbett et al., 1992). Thus, the 30 and 66 kDa proteins were thought to correspond to the *UreA* and *UreB* gene products, respectively. Interestingly an extract of *E. coli* cells harboring the recombinant plasmid pILL763 (Cussac et al., 1992) containing the *Helicobacter pylori* *UreA* and *UreB* genes, expressed two polypeptides with approximate molecular sizes of 30 and 62 kDa, which cross-reacted with the anti-*H. felis* antisera (Fig. 2B).

Table 1. Mutagenesis of *E. coli* clones and effect on urease activity.

plasmids ^a	Urease activity ^b (mmol urea min ⁻¹ mg ⁻¹ protein)
pILL205	1.2 ± 0.46 ^c
pILL205 :: a	neg ^d
pILL205 :: b	0.74 ± 0.32
pILL205 :: c	neg
pILL205 :: d	neg
pILL205 :: e	0.54 ± 0.15
pILL205 :: f	neg
pILL205 :: g	neg
pILL205 :: h	1.05 ± 0.25
pILL205 :: i	0.93 ± 0.35

a *E. coli* cells harbored pILL205 and its derivatives constructed by transposon mutagenesis. The letters correspond to the insertion sites of the MiniTn3-transposon on pILL205.

b Activities of bacteria grown aerobically for 3 days at 37°C on solid M9 minimal medium supplemented with 10 mM L-arginine. The values represent the means ± standard deviations calculated from three determinations.

c Urease activity was approximately a fifth as large as that of *H. felis* wild-type strain (ATCC 49179), i.e. 5.7 ± 0.1 mmol urea min⁻¹ protein (Ferrero and Lee, 1991).

d No activity detected (limit of detection was $< 1 \text{ nmol urea min}^{-1} \text{ mg}^{-1}$ of bacterial protein).

Clones harboring the mutated derivatives of pILL205, in all but one case, expressed the *UreA* and *UreB* gene products (Figs. 2A, B). Given that several of the mutants (i.e.; mutants "c", "d", "f" and "g") synthesized the urease subunits yet did not produce an active enzyme, it is possible to speculate that accessory functions essential for urease activity may have been disrupted by transposon insertion. In contrast, the mutant designated pILL205::a did not produce the *UreB* product and was urease-negative. Thus, the site of transposon insertion was presumed to be located in the *UreB* gene. Sequence analyses of the DNA region corresponding to insertion site "a" were undertaken to elucidate potential open reading frames encoding the structural polypeptides of *H. felis* urease.

4. Sequence analyses of *H. felis* structural urease genes:

Sequencing of a 2.4 kb region of *H. felis* DNA adjacent to transposon insertion site "a" resulted in the identification of two open reading frames (ORFs) designated *UreA* and *UreB*, which are transcribed in the same direction (Fig. 3). The transposon was confirmed to be located at 240 bp upstream from the end of *UreB*. Both ORFs commenced with an ATG start codon and were preceded by a site similar to the *E. coli* consensus ribosome-binding sequence (Shine and Dalgarno, 1974). The intergenic space for the *H. felis* structural genes consisted of three codons, which were in phase with the adjacent open reading frames. This suggests that, as has already been observed to be the case for *Helicobacter pylori* (Labigne et al., 1991), a single mutation in the stop codon of the *ure A* gene would theoretically result in a fused single polypeptide.

The *H. felis* *UreA* and *UreB* genes encode polypeptides with calculated molecular weights of 26,074 Da and 61,663 Da, respectively, which are highly

homologous at the amino acid sequence level to the *UreA* and *UreB* gene products of *H. pylori*. The levels of identity between the corresponding *ure A* and *ure B* gene products of the two *Helicobacter* spp. was calculated to be 73.5% and 88.2%, respectively. From the amino acid sequence information, the predicted molecular weights of the *UreA* and *UreB* polypeptides from *H. felis* and *H. pylori* (Labigne et al., 1991) are very similar. Nevertheless the *UreB* product of *H. felis* had a lower mobility than the corresponding gene product from *Helicobacter pylori* when subjected to SDS-polyacrylamide gel electrophoresis (Fig. 2B)

II. Expression of Recombinant Urease Subunit Proteins From *H. Pylori* and *H. Felis*: Assessment of These Proteins as Potential Mucosal Immunogens in a Mouse Model:

The aims of the study were to develop recombinant antigens derived from the urease subunits of *H. pylori* and *H. felis*, and to assess the immunoprotective efficacies of these antigens in the *H. felis*/mouse model. Each of the structural genes encoding the respective urease subunits from *H. pylori* and *H. felis* was independently cloned and over-expressed in *Escherichia coli*. The resulting recombinant urease antigens (which were fused to a 42 kDa maltose-binding protein of *E. coli*) were purified in large quantities from *E. coli* cultures and were immunogenic, yet enzymatically inactive. The findings demonstrated the feasibility of developing a recombinant vaccine against *H. pylori* infection.

A. Experimental Procedures For Part II:

1. Bacterial strains, plasmids and growth conditions:

H. felis (ATCC 49179) was grown on a blood agar medium containing blood agar base no. 2 (Oxoid) supplemented with 10% lysed horse blood

(BioMérieux) and an antibiotic supplement consisting of vancomycin (10 µg/mL), polymyxin B (25 ng/mL), trimethoprim (5 µg/mL) and amphotericin B (25 µg/mL).

Bacteria were cultured under microaerobic conditions at 37° C for 2 days, as described previously. *E. coli* strains MC1061 and JM101, used in cloning and expression experiments, were grown routinely at 37° C in Luria medium, with or without agar added. The antibiotics carbenicillin (100 µg/mL) and spectinomycin (100 µg/mL) were added as required.

2. DNA manipulations and analysis:

All DNA manipulations and analyses, unless mentioned otherwise, were performed according to standard procedures. Restriction and modification enzymes were purchased from Amersham (France). DNA fragments to be cloned were electroeluted from agarose gels and then purified by passage on Elutip mini-columns (Schleicher and Schull, Germany). Single-stranded DNA sequencing was performed using M13mp18 and M13mp19 bacteriophage vectors (Pharmacia, France). Single-stranded DNA templates were prepared from recombinant phage DNA by polyethylene glycol treatment. Sequencing of the templates was achieved according to the dideoxynucleotide chain termination method using a Sequenase kit (United States Biochemical Corp., U.S.A.).

3. Preparation of inserts for cloning using the polymerase chain reaction (PCR):

To clone the *UreA* genes of *H. pylori* and *H. felis*, degenerate 36-mer primers were conceived from the published urease sequences (Labigne et al., 1991; Ferrero and Labigne, 1993) (primer set #1; refer to Table 2). Purified DNA from *E. coli* clones harboring plasmids pILL763 and pILL207 (Table 3), that encoded the structural genes of *H. pylori* and *H. felis* ureases, were used as template material in PCR reactions. Reaction samples contained: 10 - 50 ng of denatured DNA; PCR buffer (50 mmol/L KC1 in 10 mmol/L Tris-HCl [pH 8.3]); dATP, dGTP, dCTP and dTTP (each at a final concentration of 1.25 mmol/L); 2.5

mmol/L $MgCl_2$; 25 pmol of each primer and 0.5 μ L *Taq* polymerase. The samples were subjected to 30 cycles of the following program: 2 min at 94° C, 1 min at 40° C.

The amplification products were cloned into the cohesive ends of the pAMP vector (Fig. 1) according to the protocol described by the manufacturer ("CloneAmp System", Gibco BRL; Cergy Pontoise, France). Briefly, 60 ng of amplification product was directly mixed in a buffer (consisting of 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1% (wt/vol) gelatine in 10 mmol/L Tris-HCl, pH 8.3) with 50 ng of the pAMP 1 vector DNA and 1 unit of uracil DNA glycosylase. Ligation was performed for 30 min at 37° C. Competent cells (200 μ L) of *E. coli* MC1061 were transformed with 20 μ L of the ligation mixture. Inserts were subsequently excised from the polylinker of the pAMP vector by double digestion with *Bam*HI and *Pst*I, and then subcloned into the expression vector pMAL (New England Biolabs Inc., Beverly, USA) chosen for the production of recombinant antigens (pILL919 and pILL920, respectively, Fig. 13), as well as in M13mp bacteriophage for sequencing.

Amplification of a product containing the *UreB* gene of *H. pylori* was obtained by PCR using a couple of 35-mer primers (set #2, Table 2). The PCR reaction mixtures were first denatured for 3 min at 94° C, then subjected to 30 cycles of the following program: 1 min at 94° C, 1 min at 55° C, and 2 min at 72° C. The purified amplification product (1850 bp) was digested with *Eco*RI and *Pst*I and then cloned into pMAL (pILL927, Fig. 2). Competent cells of *E. coli* MC1061 were transformed with the ligation reaction.

H. felis UreB was cloned in a two-step procedure that allowed the production of both complete and truncated versions of the *UreB* subunit. Plasmid pILL213 (Table 3) was digested with the enzymes *Dra*I, corresponding to amino acid residue number 219 of the *UreB* subunit and *Hind*III. The resulting 1350 bp fragment was purified and cloned into pMAL that had been digested with *Xmn*I

and *Hind*III (pILL219, Fig. 2). In order to produce a clone capable of synthesizing a complete UreB protein, PCR primers were developed (set #3, Table 2) that amplified a 685 bp fragment from the N-terminal portion of the *ureB* gene (excluding the ATG codon), that also overlapped the beginning of the insert in plasmid pILL219. The PCR amplified material was purified and digested with *bam*HI and *Hind*III, and then cloned into pMAL (pILL221, Figure 14). A 1350 bp *Pst*I-*Pst*I fragment encoding the remaining portion of the UreB gene product was subsequently excised from pILL219 and cloned into a linearized preparation of pILL221 (pILL222, Fig. 14).

4. Expression of recombinant urease polypeptides in the vector pMAL:

The expression vector pMAL is under the control of an inducible promoter (P_{lac}) and contains an open-reading frame (ORF) that encodes the production of MalE (Maltose-binding protein, MBP). Sequences cloned in-phase with the latter ORF resulted in the synthesis of MBP-fused proteins, which were easily purified on amylose resin. Of the two versions of pMAL that are commercially available, the version not encoding a signal sequence (*i.e.*, pMAL-c2) synthesized greater amounts of recombinant proteins and was thus used throughout.

E. coli clones harboring recombinant plasmids were screened for the production of fusion proteins prior to performing large-scale purification experiments.

5. Purification of recombinant urease polypeptides:

Fresh 500 mL volumes of Luria broth containing carbenicillin (100 μ g/mL and 2% (wt/vol) glucose were inoculated with overnight cultures (5 mL) of *E. coli* clones. The cultures were incubated at 37° C and shaken at 250 rpm, until the $A_{600} = 0.5$. Prior to adding 1 mmol/L (final concentration) isopropyl- β -D-thiogalacto-pyranoside (IPTG) to cultures, a 1.0 mL sample was taken (non-

induced cells). Cultures were incubated for a further 4 h at which time another 1.0 mL sample (induced cells) was taken. The non-induced and induced cell samples were later analyzed by SDS-PAGE.

IPTG-induced cultures were centrifuged at 7000 rpm for 20 min at 4° C and the supernatant discarded. Pellets were resuspended in 50 mL column buffer (200 mmol/L NaCl, 1 mmol/L EDTA in 10 mmol/L Tris HCl, pH 7.4), containing the following protease inhibitors (supplied by Boehringer, Mannheim, Germany): 2 µmol/L leupeptin, 2 µmol/L pepstatin, and 1 mmol/L phenylmethylsulphonyl fluoride (PMSF). Intact cells were lysed by passage through a French Pressure cell (16,000 lb/in²). Cell debris was removed by centrifugation and lysates were diluted in column buffer to give a final concentration of 2.5 mg protein/mL, prior to chromatography on a 2.6 cm x 20 cm column of amylose resin (New England Biolabs). The resin was washed with column buffer at 0.5 mL/min until the A₂₈₀ returned levels. The MBP-fused recombinant proteins were eluted from the column by washing with column buffer containing 10 mmol/L *p*-maltose.

Fractions containing the recombinant proteins were pooled and then dialyzed several times at 4° C against a low salt buffer (containing 25 mmol/L NaCl in 20 mmol/L TrisHCl, pH 8.0). The pooled fractions were then loaded at a flow rate of 0.5 mL/min onto a 1.6 x 10 cm anion exchange column (HP-Sepharose, Pharmacia, Sweden) connected to a Hi-Load chromatography system (Pharmacia). Proteins were eluted from the column using a salt gradient (25 mmol/L to 500 mmol/L NaCl). Fractions giving high absorbance readings at A₂₈₀ were exhaustively dialyzed against distilled water at 4° C and analyzed by SDS-PAGE.

6. Rabbit antisera:

Polyclonal rabbit antisera was prepared against total cell extracts of *H. pylori* strain 85P (Labigne et al., 1991) and *H. felis* (ATCC 49179). Polyclonal

rabbit antisera against recombinant protein preparations of *H. pylori* and *H. felis* urease subunits was produced by immunizing rabbits with 100 mg of purified recombinant protein in Freund's complete adjuvant (Sigma). Four weeks later, rabbits were booster-immunized with 100 µg protein in Freund's incomplete adjuvant. On week 6, the animals were terminally bled and the sera kept at -20° C.

7. Protein analyzes by SDS-PAGE and Western blotting:

Solubilized cell extracts were analyzed on slab gels comprising a 4.5% acrylamide stacking gel and a 10% resolving gel, according to the procedure of Laemmli. Electrophoresis was performed at 200 V on a mini-slab gel apparatus (Bio-Rad, USA).

Proteins were transferred to nitrocellulose paper in a Mini Trans-Blot transfer cell (Bio-Rad) set at 100 V for 1 h, with cooling. Nitrocellulose membranes were blocked with 5% (wt/vol) casein (BDH, England) in phosphate-buffered saline (PBS, pH 7.4) with gentle shaking at room temperature for 2 h. Membranes were reacted at 4° C overnight with antisera diluted in 1% casein prepared in PBS. Immunoreactants were detected using specific biotinylated secondary antibodies and streptavidin-peroxidase conjugate (Kirkegaard and Parry Lab., Gaithersburg, USA). Reaction products were visualized on autoradiographic film (Hyperfilm, Amersham, France) using a chemiluminescence technique (ECL system, Amersham).

Protein concentrations were determined by the Bradford assay (Sigma Chemicals corp., St Louis, USA).

8. Animal experimentation:

Six week old female Swiss Specific Pathogen-Free (SPF) mice were obtained (Centre d'Elevage R. Janvier, Le-Genest-St.-Isle, France) and maintained on a commercial pellet diet with water *ad libitum*. The intestines of

the animals were screened for the absence of *Helicobacter muridarum*. For all orogastric administrations, 100 μ L aliquots were delivered to mice using 1.0 mL disposable syringes to which polyethylene catheters (Biotrol, Paris, France) were attached.

Preparation of sonicated extracts and inocula from *H. felis* res: _____

H. felis bacteria were washed in PBS and centrifuged at 5000 rpm, for 10 min in a Sorvall RC-5 centrifuge (Sorvall, USA) at 4° C. The pellets were washed twice and resuspended in PBS. Bacterial suspensions were sonicated as previously described and were subjected to at least one freeze-thaw cycle. Protein determinations were carried out on the sonicates.

To ensure a virulent culture of *H. felis* for protection studies, *H. felis* bacteria were maintained *in vivo* until required. Briefly, mice were inoculated three times (with 10^{10} bacteria/mL), over a period of 5 days. The bacteria were reisolated from stomach biopsies on blood agar medium (4 - 7 days' incubation in a microaerobic atmosphere at 37° C). Bacteria grown for two days on blood agar plates were harvested directly in peptone water (Difco, USA). Bacterial viability and motility were assessed by phase microscopy prior to administration to animals.

10. Mouse proton studies:

Fifty mg of recombinant antigen and 10 μ g cholera holotoxin (Sigma Chemical Corp.), both resuspended in HCO_3^- , were administered orogastrically to mice on weeks 0, 1, 2 and 3. Mice immunized with sonicated *H. felis* extracts (containing 400 - 800 μ g of total protein) were also given 10 μ g of cholera toxin. On week 5, half of the mice from each group were challenged with an inoculum of virulent *H. felis*. The remainder of the mice received an additional "boost" immunization on week 15. On week 17 the latter were challenged with a culture of *H. felis*.

11. Assessment of *H. felis* colonization of the mouse:

Two weeks after receiving the challenge dose (i.e., weeks 7 and 19, respectively) mice were sacrificed by spinal dislocation. The stomachs were washed twice in sterile 0.8% NaCl and a portion of the gastric antrum from each stomach was placed on the surfaces of 12 cm x 12 cm agar plates containing a urea indicator medium (2% urea, 120 mg Na₂HPO₄, 80 mg KH₂PO₄, 1.2 mg phenol red, 1.5 g agar prepared in 100 mL). The remainder of each stomach was placed in formal-saline and stored until processed for histology. Longitudinal sections (4 µm) of the stomachs were cut and routinely stained by the Giemsa technique. When necessary, sections were additionally stained by the Haematoxylin-Eosin and Warthin-Starry silver stain techniques.

The presence of *H. felis* bacteria in mouse gastric mucosa was assessed by the detection of urease activity (for up to 24 h) on the indicator medium, as well as by the screening of Giemsa-stained gastric sections that had been coded so as to eliminate observer bias. The numbers of bacteria in gastric sections were semi-quantitatively scored according to the following scheme: 0, no bacteria seen throughout sections; 1, few bacteria (< 20) seen throughout; 2, occasional high power (H.P.) field with low numbers (< 20) of bacteria; 3, occasional H.P. field with low to moderate numbers (< 50) of bacteria; and 4, numerous (> 5) H.P. fields with high numbers of bacteria (> 50). Mononuclear cell infiltrates were scored as follows: 0, no significant infiltration; 1, infiltration of low numbers of mononuclear cells limited to the submucosa and muscularis mucosa; 2, infiltration of moderate numbers of mononuclear cells to the submucosa and muscularis mucosa, sometimes forming loose aggregates; and 3, infiltration of large numbers of mononuclear cells and featuring nodular agglomerations of cells.

B. Results Of Part II Experiments:

1. Expression of *Helicobacter urease* polypeptides in *E. coli*:

Fragments containing the sequences encoding the respective *UreA* gene products of *H. felis* and *H. pylori* were amplified by PCR and cloned in-phase with an ORF encoding the 42 kDa MBP, present on the expression vector pMAL. Sequencing of the PCR products revealed minor nucleotide changes that did not, however, alter the deduced amino acid sequences of the respective gene products. *E. coli* MC1061 cells transformed with these recombinant plasmids (pILL919 and pILL920, respectively) expressed fusion proteins with predicted molecular weights of approximately 68 kDa. Following chromatography on affinity (amylose resin) and anion exchange gel media (Q-Sepharose), these proteins were purified to high degrees of purity (Fig. 1). The yield from 2-L cultures of recombinant *E. coli* cells was approximately 40 mg of purified antigen.

Similarly, the large *UreB* subunits of *H. pylori* and *H. felis* ureases were expressed in *E. coli* (plasmids pILL927 and pILL222, respectively) and produced fusion proteins with predicted molecular weights of 103 kDa. The yield in these cases was appreciably lower than for the *UreA* preparations (approximately 20 mg was recovered from 2-L of bacterial culture). Moreover, problems associated with the cleavage of the *UreB* polypeptides from the MBP portion of the fusion proteins were encountered. These difficulties were attributed to the large sizes of the recombinant *UreB* polypeptides.

2. Analysis of the recombinant urease polypeptides:

Western blot analyses of the antigen preparations with rabbit polyclonal antisera raised to whole-extracts of *H. pylori* and *H. felis* bacteria demonstrated that the antigens retained immunogenicity to the homologous as well as heterologous antisera (Figs. 14 and 15). The antisera did not recognize the MBP

component alone. Cross-reactivity between the urease polypeptides of *H. pylori* and *H. felis* was consistent with the high degrees of identity between the amino acid sequences of these proteins.

Rabbit polyclonal antisera raised against purified recombinant UreA and UreB proteins prepared from *H. pylori* and *H. felis* strongly reacted with the urease polypeptides present in whole-cell extracts of the bacteria (Fig. 16). As we had already observed, the UreB subunit of *H. felis* urease migrated slightly higher on SDS-PAGE gels than did that of *H. pylori* (Fig. 16).

3. Preparation of *H. felis* inocula used in immunoprotection studies:

To ensure the virulence of *H. felis* bacterial inocula, bacteria were reisolated from *H. felis*-infected mouse stomachs (see Materials and Methods). The bacteria were passaged a minimum number of times *in vitro*. Stock cultures prepared from these bacteria, and stored at -80° C, were used to prepare fresh inocula for other mouse protection studies. This procedure ensured that the inocula used in successive experiments were reproducible.

Immunization of mice against gastric *H. felis* infection:

Mice that had been immunized for three weeks with the given antigen preparations were divided into two lots and one half of these were challenged two weeks later with an *H. felis* inoculum containing 10^7 bacteria/mL. One group of animals that had been immunized with recombinant *H. felis* UreA were also challenged but, unlike the other animals, were not sacrificed until week 19.

a) Protection at week 5:

Eighty-five % of stomach biopsy samples from the control group of mice immunized with *H. felis* sonicate preparations were urease-negative and therefore appeared to have been protected from *H. felis* infection (Table 4). This compared to 20% of those from the other control group of animals given MBP alone. The proportion of urease-negative stomachs for those groups of mice

given the recombinant urease subunits varied from 70% (for *H. pylori* UreB) to 20% (for *H. pylori* UreA).

The levels of bacterial colonization by *H. felis* was also assessed from coded histological slides prepared from gastric tissue. Due to the striking helical morphology of *H. felis* bacteria, the organisms could be readily seen on the mucosal surfaces of both gastric pit and glandular regions of the stomach. Histological evidence indicated that the levels of protection in mice was lower than that observed by the biopsy urease test: 25% and 20% of gastric tissue from mice immunized with *H. felis* sonicate preparations of *H. pylori* UreB, respectively, were free of *H. felis* bacteria.

Amongst certain groups of these mice the preponderance of urease-negative biopsies, as well as lower histological scores for bacterial colonization (unpublished data), suggested that an immunoprotective response had been elicited in the animals. This response, however, may have been insufficient to protect against the inoculum administered during the challenge procedure.

b) Protection at week 17:

The remaining mice, from each group of animals, were boosted on week 15. These mice were challenged at week 17 with an *H. felis* inoculum containing approximately 100-fold less bacteria than that used previously. Two weeks later all stomach biopsies from the MBP-immunized mice were urease-positive (Table 4). In contrast, urease activity for gastric biopsies from mice immunized with the recombinant urease subunits varied from 50% for *H. pylori* UreA to 100% for *H. felis* UreB. The latter was comparable to the level of protection observed for the group of animals immunized with *H. felis* sonicated extracts. Histological evidence demonstrated that the UreB subunits of *H. felis* and *H. pylori* protected 60% and 25% of immunized animals, respectively. This compared with a level of 85% protection for mice immunized with *H. felis* sonicated extracts. Immunization of mice with recombinant *H. pylori* UreA did not

protect the animals. Similarly, the stomachs of all *H. felis* UreA-immunized mice, that had been challenged at week 5, were heavily colonized with *H. felis* bacteria at week 19 (Table 4).

The urease gastric biopsy test, when compared to histological analysis of gastric tissue sections, gave sensitivity and specificity values of 63% and 95%, respectively. Thus, histology proved to be the more accurate predictor of *H. felis* infection in the mouse.

5. Cellular immune response in immunized stomachs:

In addition to the histological assessment of *H. felis* colonization, mouse gastric tissue was also scored (from 0 to 3) for the presence of a mononuclear cell response. In mice immunized with MBP alone, a mild chronic gastritis was seen with small numbers of mononuclear cells restricted to the muscularis mucosa and to the submucosa of the gastric epithelium. In contrast, there were considerable numbers of mononuclear cells present in the gastric mucosae from animals immunized with either the recombinant urease polypeptides, or with *H. felis* sonicate preparations. These inflammatory cells coalesced to form either loose aggregates, in the submucosal regions of the tissue, or nodular structures that extended into the mucosal regions of the gastric epithelia. The mononuclear cell response did not appear to be related to the presence of bacteria as the gastric mucosae from the *H. felis* UreA-immunized mice, that were heavily colonized with *H. felis* bacteria, contained little or no mononuclear cells.

Table 2. The oligomeric primers used in PCR-based amplification of urease-encoding nucleotide sequences.

Primer set	Nucleotide sequence (5' ->3')
#1 forw	...CAU CCT ^T AAA ^G GAA ^G T ^C TA ^T GAT ^C AAA ^G T ^C TA ^T ATG
rev	T ^C TC C ^T TT A ^T CG A ^T CG A ^T G ^C A ^T A ^G .TAT C ^T TT C ^T TT CAT CUA...
#2 forw	CC GGA <u>GAA TTC</u> ATT AGC AGA AAA GAA TAT GTT TCT ATG <i>EcoRI</i> ¥
rev	AC GTT <u>CTG CAG</u> CTT ACG AAT AAC TTT TGT TGC TTG AGC <i>PstI</i> ¥
#3 forw	<u>GGA TCC</u> AAA AAG ATT TCA CG <i>BamHI</i> ¥
rev	GGA AGC TT C <u>TGC AGG</u> TGT GCT TCC CCA GTC <i>HindIII</i> ¥ <i>PstI</i> ¥

* Degenerated nucleotides in which all possible permutations of the genetic code were included (A, T, G, C).

G,C,T The given nucleotides were degenerate with the specific base(s) shown.

¥ Restriction sites introduced in the amplified fragments.

Table 3. Plasmids used.

Plasmid	Vector	Relevant phenotype or character	Reference
pILL763	pILL570	9.5 kb fragment (Sau3a partial digest of <i>H. pylori</i> chromosome)(Sp ^R)	Cussac et al., 1991
pILL199	pILL575	35 kb fragment (Sau3A partial digest of <i>H. felis</i> chromosome)	Ferrero & Labigne, '93
pILL207	pILL570	11 kb fragment (Sau3A partial digest of pILL199)	Infection & Immunity 1994, 62: 4981-4989
pILL919	pMAL- C2	0.8 kb BamHI-PstI ^a insert containing a nucleotide fragment encoding <u><i>H. felis</i></u> ureA gene (Ap ^R)	Infection & Immunity 1994, 62: 4981-4989

Plasmid	Vector	Relevant phenotype or character	Reference
pILL920	pMAL-C2	0.8 kb BAMHI-PstI ^a insert containing PCR product encoding <i>H. pylori</i> ureA gene	Infection & Immunity 1994, 2: 4981-4989
pILL927	pMAL-C2	1.8 kb EcoRI-PstIa PCR fragment encoding <i>H. pylori</i> ureB gene	Infection & Immunity 1994, 62: 4981-4989
pILL213	pUC19	2 kb fragment resulting from Sau2A partial digest of pILL207 (Ap ^R)	Infection & Immunity 1994, 62: 4981-4989
pILL219	pMAL-C2	1.4 kb DraI-HindIII ^b insert containing <i>H. felis</i> ureB (bases 657 - 1707)	Infection & Immunity 1994, 62: 4981-4989

Plasmid	Vector	Relevant phenotype or character	Reference
pILL221	pMAL-C2	0.7 kb BamHI-PstI PCR fragment encoding <i>H. felis</i> ureB (bases 4 - 667)	Infection & Immunity 1994, 62: 4981-4989
pILL222	pMAL-C2	1.35 kb PstI-HindIII fragment encoding <i>H. felis</i> ureN (bases 667 - 1707) from pILL219 cloned into linearized pILL221	Infection & Immunity 1994, 62: 4981-4989

Table 4. Protection of mice by immunization with recombinant urease proteins.

Antigen	Protection (%) ^a			
	Urease		Histology	
MBP	0 %	(0/10)	0 %	(0/10)
UreA <i>H. pylori</i>	50	(4/8)	0	(0/10)
UreA <i>H. felis</i> ^b	12.5	(1/8)	0	(0/10)
UreB <i>H. pylori</i>	65	(5/8)	25	(2/8)
UreB <i>H. felis</i>	100	(7/7)	60	(5/7)
<i>H. felis</i> sonicate	100	(8/8)	85	(7/8)

a Challenge inoculum dose was 10^5 bacteria/mouse

b Mice were challenged on week 5 (with 10^7 bacteria) and were sacrificed on week 19.

III. *Helicobacter Pylori* HspAB Heat Shock Gene Cluster: Nucleotide Sequence, Expression And Function:

A homolog of the heat shock proteins (Hsps) of the GroEL class, reported to be closely associated with the disease of *Helicobacter pylori* (a nickel metalloenzyme), has recently been purified from *H. pylori* cells by Dunn et al., and others et al. (Infect. Immun. 60:1946, 1992, 1946 and 2125, respectively). Based on the reported N-terminal amino acid sequence of this immunodominant protein, oligonucleotides were synthesized in order to target the gene (*hspB*) encoding the GroEL-like protein in the chromosome of *H. pylori* strain 85P. Following gene amplification, a 108-base pair (bp)-fragment encoding the 36 first amino acids of the *hspB* protein was purified, and used a probe to identify in the *H. pylori* genomic bank a recombinant cosmid harboring the entire *hspB* encoding gene. The *hspB* gene was mapped to a 3.15 kilobases (kb) *Bgl*II restriction fragment of the pILL684 cosmid (Table 5). The nucleotide sequence of that fragment subcloned into the pILL570 plasmid vector (pILL689) revealed the presence of two open reading frames (ORFs) designated *hspA* and *hspB*, the organization of which was very similar to be groESL bicistronic operons of other bacterial species. *hspA* and *hspB* encode polypeptides of 118 and 545 amino acids, respectively, corresponding to calculated molecular masses of 13.0 and 58.2 kilodaltons (kDa), respectively. Amino acid sequence comparison studies revealed i) that the *H. pylori* HspA and HspB protein were highly similar to their bacterial homologs; ii) that the HspA *H. pylori* protein features a striking motif at the carboxyl terminus that other bacterial GroEs-homologs lack; this unique motif consists of a series of eight histidine residues resembling metal binding domain, such a nickel binding. Surprisingly, immediately upstream of the gene cluster an IS5 insertion element was found that was absent in the *H. pylori* genome, and was positively selected during the cosmid cloning process. The IS5 was found to be involved in the expression of the *hspA* and *hspB* genes in pILL689. The

expression of the HspA and HspB proteins from the pILL689 plasmid was analyzed in minicell-producing strain. Both polypeptides were shown to be constitutively expressed in the *E. coli* cells. When the pILL689 recombinant plasmid was introduced together with the *H. pylori* urease gene cluster into an *E. coli* host strain, an increase of urease activity was observed suggesting a close interaction between the heat shock proteins and the urease enzyme. Supporting the concept of a specific function for the HspA chaperone, was the fact that whereas a single *hspB* copy was found in the *H. pylori* genome, two copies of the *hspA* were found in the genome, one linked to the *hspB* gene and one unlinked to the *hspB* gene. Attempts to construct isogenic mutants of *H. pylori* in the *HspA* and the *hspB* gene were unsuccessful suggesting that these genes are essential for the survival of the bacteria.

A. Experimental Procedures For Part III:

1. Bacterial strains, plasmids, and culture conditions:

The cloning experiments were performed with genomic DNA prepared from *H. pylori* strain N6 deposited at NCIMB No. 40512 on June 26, 1992 as the recipient strain for the electroporation experiments because of its favorable transformability. *E. coli* strain HB101 or strain MC1061 were used as a host for cosmid cloning and subcloning experiments, respectively. *E. coli* P678-54 was used for preparation of minicells.

Vectors and recombinant plasmids used in this study are listed in Table 1. *H. pylori* strains were grown on horse blood agar plates, supplemented with vancomycin (10 mg/l), polymyxin B (2,500 U/l), trimethoprim (5 mg/l), and amphotericin B (4 mg/l). Plates were incubated at 37°C under microaerobic conditions in an anaerobic jar with a carbon dioxide generator envelope (BBL 70304). *E. coli* strains were grown in L-broth without glucose (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter; pH 7.0) or on L-agar plates (1.5% agar) at 37°C. For measurement of urease activity, the nitrogen-limiting medium used consisted of ammonium-free M9 minimal agar medium (pH 7.4) containing 0.4% D-glucose as the carbon source, and freshly prepared filter-sterilized L-arginine added to the final concentration of 10 mM. Antibiotic concentrations for the selection of recombinant clones were as follows (in milligrams per liter): kanamycin, 20; spectinomycin, 100; carbenicillin, 100.

2. Preparation of DNA:

Genomic DNA from *H. pylori* was prepared as previously described. Cosmid and plasmid DNAs were prepared by an alkaline lysis procedure followed by purification in cesium chloride-ethidium bromide gradients as previously described.

3. Cosmid cloning:

The construction of the cosmid gene bank of *H. pylori* 85P in *E. coli* HB101, which was used for the cloning of the *H. pylori* *HspA-B* gene cluster, has been described previously. Labigne et al., 1991, J. Bact. 173:1920.

4. DNA analysis and cloning methodology:

Restriction endonucleases, T4 DNA ligase, DNA polymerase I large (Klenow) fragment, and *Taq* polymerase were purchased from Amersham, T4 DNA polymerase from Biolabs, and calf intestinal phosphatase from Pharmacia. All enzymes were used according to the instructions of the manufacturers. DNA fragments were separated on agarose gels run in Tris-acetate buffer. The 1-kb ladder from Bethesda Research Laboratories was used as a fragment size standard. When necessary, DNA fragments were isolated by electroelution from agarose gels as previously described and recovered from the migration buffer by means of an Elutip-d minicolumn (Schleicher and Schuell, Dassel, Germany). Basic DNA manipulations were performed according to the protocols described by Sambrook et al.

5. Hybridization:

Colony blots for screening of the *H. pylori* cosmid bank and for identification of subclones were prepared on nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) according to the protocol of Sambrook et al. Radioactive labelling of PCR-products was performed by random priming using as primers the random hexamers from Pharmacia. Colony hybridizations were performed under high stringency conditions (5 x SSC, 0.1% SDS, 50% formamide, 42° C) (1 x SSC; 150 mM NaCl, 15 mM sodium citrate, pH 7.0). For Southern blot hybridizations, DNA fragments were transferred from agarose gels to nitrocellulose sheets (0.45 - μ m pore size; Schleicher & Schuell, Inc.), and hybridized under low stringency conditions (5 x SSC, 0.1% SDS, 30 or

40% formamide, at 42° C with ³²P-labeled deoxyribonucleotide probes.) Hybridization was revealed by autoradiography using Amersham Hyperfilm-MP.

6. DNA sequencing:

Appropriate fragments of plasmid DNA were subcloned into M13 mp 18/19 vectors. Single-stranded DNA was prepared by phage infection of *E. coli* strain JM101. Sequencing was performed by the dideoxynucleotide chain termination method using the United States Biochemicals Sequenase kit. Both the M13 universal primer and additional specific primers (Fig. 1) were used to sequence both the coding and non-coding DNA strands. Sequencing of double-stranded DNA was performed as previously described. Direct sequencing of PCR product was carried out following purification of the amplified, electroeluted PCR product through an Elutip-d minicolumn (Schleicher & Schuell). The classical protocol for sequencing using the Sequenase kit was then used with the following modifications: PCR product was denatured by boiling annealing mixture containing 200 picomoles of the oligonucleotide used as primer and DMSO to the final concentration of 1% for 3 minutes; the mixture was then immediately cooled on ice; the labeling step was performed in presence of manganese ions (mM).

7. Electroporation of *H. pylori*:

In the attempt to construct *H. pylori* mutants, appropriate plasmid constructions carrying the targeted gene disrupted by a cassette containing a kanamycin resistance gene (*aph3'*-III), were transformed into *H. pylori* strain N6 by means of electroporation as previously described. Plasmid pSUS10 harboring the kanamycin disrupted *flaA* gene was used as positive control of electroporation. After electroporation, bacteria were grown on non-selective plates for a period of 48 h in order to allow for the expression of the antibiotic resistance and then transferred onto kanamycin-containing plates. The selective plates were incubated for up to 6 days.

8. Polymerase chain reaction (PCR):

PCRs were carried out using a Perkin-Elmer Cetus thermal cycler using the GeneAmp kit (Perkin-Elmer Cetus). Classical amplification reaction involved 50 picomoles (pmoles) of each primer and at least 5 pmoles of the target DNA. The target DNA was heat denatured prior to addition to the amplification reaction. Reaction consisted of 25 cycles of the following three steps: denaturation (94° C for 1 minute), annealing (at temperatures ranging between 42° and 55° C, depending on the calculated melting temperatures of the primers, for 2 min), and extension (72° C for 2 min). When degenerate oligonucleotides were used in nonstringent conditions, up to 1000 pmoles of each oligonucleotide were added, 50 cycles were carried out, and annealing was performed at 42° C.

9. Analysis of proteins expressed in minicells:

Minicells harboring the appropriate hybrid plasmid were isolated and labeled with [³⁵S] methionine (50 μ -m Ci/ml). Approximately 100,000 cpm of acetone-precipitable material was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in a 12.5% gel. Standard proteins with molecular weights ranging from 94,000 to 14,000 (low molecular-weights kit from Bio-Rad Laboratories) were run in parallel. The gel was stained and examined by fluorography, using En³Hance (New England Nuclear).

10. Urease activity:

Urease activity was quantitated by the Berthelot reaction by using a modification of the procedure, which has already been described (Cussac et al., 1992, J. Bact. 176:2666-2673). Urease activity was expressed as micromoles of urea hydrolyzed per minute per milligram of bacterial protein.

B. Results Of Part III Experiments:

1. Identification of a recombinant cosmid harboring the *Helicobacter pylori* GroEL-like heat shock protein encoding gene:

Based on the published N-terminal amino sequence of the purified heat shock protein of *H. pylori*, two degenerate oligonucleotides were synthesized to target the gene of interest in the chromosome of *H. pylori* strain 85P. The first one 5' - **G C N A A R G A R A T H A A R T T Y T C N G** - 3', where N stands for the four nucleotides, R = A and G, Y = T and C, H = T, C, and A, is derived from the first 8 amino acids of the protein (AKEIKFSD); the second one 5' - **C R T T N C K N C C N C K N G G N C C C A T** - 3', where K = G and T, corresponds to the complementary codons specifying the amino acid from position 29 to position 36 (MGPRGRNV, Evans et al. 1992, Inf. Immun., 60:2125-2127). The expected size for the PCR product was 108 base pairs (bp). The amplification reaction was performed under low stringency conditions as described in the Materials and Methods section, and led to the synthesis of six fragments with sizes ranging from 400 bp to 100 bp. The three smallest fragments were electroeluted from an acrylamide gel and purified. Direct sequencing of the PCR products permitted the identification of a DNA fragment encoding an amino acid sequence corresponding to the published sequence. This fragment was, therefore, labeled and used as probe in colony hybridization to identify recombinant cosmids exhibiting homology to a 5' segment of the *H. pylori* GroEL-like encoding gene; this gene was further designated *hspB*. The gene bank consists of 400 independent kanamycin-resistant *E. coli* transductants harboring recombinant cosmids. Of those, one single clone hybridized with the probe and harbored a recombinant plasmid designated pILL684, 46 kb in size. The low frequency observed when detecting the *hspB* gene (1 of 400) was unusual when compared with that of several cloned genes, which were consistently detected in five to seven recombinant cosmids. In order to identify the *hspB* gene, fragments with sizes of 3 to 4 kb were generated by partial restriction of the pILL684 cosmid DNA with endonuclease *Sau3A*, purified, and ligated into the *Bgl*II site of plasmid vector pILL570. Of 100 subclones, 7 were positive clones, and one was further

studied (pILL689) (Table 5); it contains a 3.15 kb insert, flanked by two *Bgl*II restriction sites, that was mapped in detail (Fig. 5). Using the PCR 32 P labeled probe, the 5' end of the *hspB* gene was found to map to the 632 bp *Hind*III-*Sph*I central restriction fragment of pILL689, indicating that one could expect the presence of the entire *HspB* gene in the pILL689 recombinant plasmid.

2. DNA sequence and deduced amino acid sequence of the *H. pylori* *HspA-B* gene cluster: _____

The 2300 bp of pILL689 depicted in Fig. 5 were sequenced by cloning into M13mp18 and M13mp19, the asymmetric restriction fragments *Bgl*II-*Sph*I, *Sph*I-*Hind*III, *Hind*III-*Bgl*II; each cloned fragment was independently sequenced on both strands, 16 oligonucleotide primers were synthesized to confirm the reading and/or to generate sequences overlapping the independently sequenced fragments; these were used as primers in double-stranded DNA sequencing analyses.

The analysis of the sequence revealed two distinct genetic elements. First the presence of two open reading frames (ORFs), depicted in Figure 5, transcribed in the same direction, that were designated *hspA* and *hspB*. The nucleotide sequence and the deduced amino acid sequence of the two ORFs are presented in Fig. 6. The first codon of *hspA* begins 323 bp upstream of the leftward *Hind*III site of pILL689 (Fig. 5) and is preceded by a Shine-Dalgarno ribosome-binding site (RBS) (GGAGAA). The *hspA* ORF codes for a polypeptide of 118 amino acids. The initiation codon for the *hspB* ORF begins 25 nucleotides downstream the *hspA* stop codon; it is preceded by a RBS site (AAGGA). *hspB* ORF encodes a polypeptide of 545 amino acids and is terminated by a TAA codon followed by a palindromic sequence resembling a rho-independent transcription terminator (free energy, $\Delta = -19.8$ kcal/mol) (Fig. 6). The N-terminal amino acid sequence of the deduced protein HspB was identical to the N-terminal sequence of the purified *H. pylori* heat shock protein previously

published with the exception of the N-terminal methionine, which is absent from the purified protein and might be post-translationally removed, resulting in a mature protein of 544 amino acids.

The deduced amino acid sequences of *H. pylori* HspA and HspB were compared to several amino acid sequences of Hsps of the GroES and GroEL class (Fig. 7). HspB exhibited high homology at the amino acid level with the *Legionella pneumophila* HtpB protein (82.9% of similarities), with the *Escherichia coli* GroEL protein (81.0% of similarities), with the *Chlamydia psittaci* or *C. trachomatis* HypB protein (79.4% of similarities), with *Clostridium perfringens* Hsp60 protein (80.7% of similarities), and to a lesser extent to the GroEL-like proteins of *Mycobacterium*. However, like almost all the GroEL homologs, *H. pylori* HspB demonstrated the conserved carboxyl-terminus glycine-methionine motif (MGGMGGMGGMGGMM), which was recently shown to be dispensable in the *E. coli* GroEL chaperonin. The degree of homology at the amino acid level between the *H. pylori* HspA protein and the other GroES-like proteins is shown in Fig. 7. The alignment shown features a striking motif at the carboxyl terminus of the *H. pylori* HspA protein that other bacterial GroES-homologs lack. This unique highly charged motif consists of 27 additional amino acids capable of forming a loop between two double cysteine residues; of the 27 amino acids, 8 are histidine residues highly reminiscent of a metal binding domain.

The second genetic element revealed by the sequence analysis, was the presence of an insertion sequence (IS5) 84 bp upstream of the *hspA* gene. The nucleotide sequence of this element matched perfectly that previously described for IS5 in *E. coli*, with the presence of a 16 nucleotide sequence (CTTGTTTCGCACCTTCC) that corresponds to one of the two inverted repeats, which flank the IS5 element. Because of the perfect match at the DNA level, we suspected that the IS5 was not initially present in the *H. pylori* chromosome, but

had rather inserted upstream of the *HspA-HspB* gene cluster during the cloning process, a hypothesis that needed to be confirmed by further analyses.

3. Identification of the upstream sequence of the *HspA-B* gene cluster in *H. pylori* chromosome:

The presence of the IS5 was examined by gene amplification using two oligonucleotides, one being internal to the IS5 element and the other one downstream of the IS5 element to target a putative sequence i) in the chromosome of *H. pylori* strain 85P, ii) in the initial cosmid pILL684 (Table 5), and iii) in the 100 subclones resulting from the *Sau3A* partial restriction of the pILL684 recombinant cosmid. IS5 was absent from the chromosome of *H. pylori*, and was present in the very first subcultures of the *E. coli* strain harboring cosmid pILL684. Among the 100 pILL684 subclone derivatives that appeared to contain all or part of the IS5 sequence, we then looked for a subclone harboring the left end side of the IS5 plus the original upstream sequence of the *HspA-HspB* gene cluster. This screening was made by restriction analysis of the different *Sau3A* partial generated subclones. The restriction map of one (pILL694) of the plasmids fulfilling these criteria is shown in Suerbaum et al., 1996, Molec. Microbiology. The left end side of the IS5 nucleotide sequence was determined; the presence of a 4-bp duplication CTAA on both sides of the 16-bp inverted repeats of the IS5 element allowed us to confirm the recent acquisition of the IS5 element by transposition. A 245-nucleotide sequence was then determined that mapped immediately upstream of the IS5 element. This sequence consists of a non-coding region in which the presence of a putative consensus heat shock promoter sequence was detected; it shows a perfectly conserved -35 region (TAACTCGCTTGAA) and a less consentaneous -10 region (CTCAATTA). Two oligonucleotides were synthesized, which mapped to sequences located on both sides of the IS5 element present in the recombinant cosmid; these two oligonucleotides should lead to the amplification of a 350bp fragment when the

polypeptides visualized on the SDS gel was in good agreement with the copy number of the respective vectors, the intensity of the two polypeptidic bands suggested a polycistronic transcription of the two genes.

5. Attempts to understand the role of the HspA and HspB proteins:

Two disruptions of genes were achieved in *E. coli* by inserting the Km cassette previously described within the *hspA* or the *hspB* gene of plasmids pILL686 and pILL691. This was done in order to return the disrupted genes in *H. pylori* by electroporation, and to select for allelic replacement. The pILL696 resulting plasmid encoded a truncated form of the HspA protein, corresponding to the deletion of the C-terminal end amino acid sequence, in that plasmid the Km cassette was inserted in such way that the promoter of the Km gene could serve as promoter for the *HspB* downstream gene. The pILL687 and pILL688 plasmids (Table 5) resulted from the insertion of the Km cassette in either orientation within the *hspB* gene. None of these constructs led to the isolation of kanamycin transformants of *H. pylori* strain N6, when purified pILL687, pILL688, pILL696 plasmids (Table 5) were used in electroporation experiments, whereas the pSUS10 plasmid used as positive control always did. These results suggest the *H. pylori* HspA and HspB protein are essential proteins for the survival of *H. pylori*.

Because of i) the constant description in the literature of a close association of the HspB protein with the urease subunits; ii) the unique structure of the HspA protein with the C-terminal sequence reminiscent of a nickel binding domain, and iii) of the absence of viable *HspA* and/or *HspB* mutants of *H. pylori*, we attempted to demonstrate a role of the *H. pylori* Hsp proteins in relation with the *H. pylori* urease by functional complementation experiments in *E. coli*. Plasmids pILL763 or pILL753 (both pILL570 derivatives, Table 5) encoding the urease gene cluster were introduced with the compatible pILL692 plasmid

(pACYC177 derivative) that constitutively expresses the HspA and HspB polypeptides as visualized in minicells. In both complementations, the expression of the HspA and HspB proteins in the same *E. coli* cell allows to observe a three-fold increase in the urease activity following induction of the urease genes on minimum medium supplemented with 10 mM L⁻¹ arginine as limiting nitrogen source.

Table 5: Vectors and hybrid plasmids used in this study.

Plasmid	Vector	Size(kb)	Characteristics(a)	Origin or Reference
	PILL575	10	Mob, Cos, Km	
	PILL570	5.3	Mob, Sp	
	PACYC177	3.9	Ap, Km	
	PBR322	5.7	Ap, Km, source of Km-cassette	
PILL600	PILL575	46	Mob, Km, cosmid containing <i>H. pylori</i> hspA-B	Sau3A partial digest of <i>H. pylori</i> 8Sp DNA
PILL604	PILL570	9.29	Mob, Sp, plasmid containing <i>H. pylori</i> hspB	Sau3A partial digest of PILL684
PILL685	PUC19+c	4.5	Ap, plasmid containing <i>H. pylori</i> hspB	1.9-kb BglII-ClaI PILL685 cloned into pUC19*
PILL686	PUC19*(c)	5.9	Ap, Km, <i>H. pylori</i> hspB [Km-orientation A(b)]	1.4-kb SmaI-SmaI PILL600 cloned into PILL686
PILL687	PUC19*(c)	5.9	Ap, Km, <i>H. pylori</i> hspB [Km-orientation B(b)]	1.4-kb SmaI-SmaI PILL600 cloned into PILL686
PILL688	PUC19*(c)	5.9	Ap, Km, <i>H. pylori</i> hspB [Km-orientation A(b)]	Sau3A partial digest of PILL684
PILL689	PILL570	8.45	Mob, Sp, plasmid containing <i>H. pylori</i> hspA-B	SphI-SphI PILL689 cloned into pUC19**
PILL691	PUC19** (c)	3.9	Ap, plasmid containing <i>H. pylori</i> hspA 1.3-kb	3.15-kb BglII PILL689 cloned into PACYC177
PILL692	PACYC177	7.05	Ap, Km, plasmid containing <i>H. pylori</i> hspA-B	Sau3A partial digest of PILL684
PILL694	PILL570	8.7	Sp, plasmid containing left end of IS5	1.4-kb SmaI-SmaI PILL600 cloned into PILL691
PILL696	PUC19** (c)	5.3	Ap, Km, <i>H. pylori</i> hspA [Km-orientation A (b)]	
PSUS10	PIC20R2	7.7	Ap, Km, <i>H. pylori</i> flaA [Km	
PILL753	PILL570	16.5	Sp, plasmid containing ureA, B, C, D, E, F, G, H, I	
PILL763	PILL570	14.75	Sp, plasmid containing ureA, B, E, F, G, H, I-	

(a) Mob, conjugative plasmid due to the presence of *oriT*; Ap, Km and Sp, resistance to ampicillin, kanamycin, and spectinomycin, respectively; Cos, presence of lambda cos site.

(b) Orientation A indicates that the Kanamycin promoter initiates transcription in the same orientation as that of the gene where the cassette has been inserted; orientation B, the opposite.

(c) PUC19* and pUC19** derivatives from pUC19 vector in which the SphI and HindIII site, respectively, have been end-filled by using the Klenow polymerase and self religated.

IV. Expression, Purification And Immunogenic Properties Of *H. Pylori* HspA AND HspB:

A. Experimental Procedure For Part IV:

Expression and purification of recombinant fusion proteins:

The MalE-HspB fusion proteins were expressed following the cloning of the two genes within the pMAL-c2 vector as described in the "Results" section using the following primers:

oligo #1 ccggagaattcAAGTTTCAACCATTAGGAGAAAGGGTC

oligo #2 acgttctgcagTTTAGTGTTTTTTGTGATCATGACAGC

oligo #3 ccggagaattcGCAAAAGAAATCAAATTTTCAGATAGC

oligo #4 acgttctgcagATGATACCAAAAAGCAAGGGGGGCTTAC

Two liters of Luria medium containing glucose (30%) and ampicillin (100 µg/ml) were inoculated with 20 ml of an overnight culture of strain MC1061 containing the fusion plasmid and incubated with shaking at 37°C. When the OD600 of the culture reached 0.5, IPTG (at a final concentration of 10 mM) was added, and the cells were incubated for a further 4 hours. Cells were harvested by centrifugation (5000 rpm for 30 min at 4°C), resuspended in 100 ml of column buffer consisting of 10 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA supplemented with protease inhibitors [(Leupeptin (2 µM) - Pepstatin (2 µM) - PMSF (1mM) - Aprotinin (1:1000 dilution)], and passed through a French press. After centrifugation (10,000 rpm for 20 min at 4°C), the supernatant were recovered and diluted (2-fold) with column buffer. The lysate was filtered through a 0.2 µm nitrocellulose filter prior to loading onto a pre-equilibrated amylose resin (22 x 2.5 cm). The fusion proteins were eluted with a 10mM maltose solution prepared in column buffer, and the fractions containing the fusion proteins were pooled, dialyzed against distilled water, and lyophilized. Fusion proteins were resuspended in distilled

water at a final concentration of 2 mg of lyophilized material/ml, and stored at -20°C. Concentration and purity of the preparations were controlled by the Bradford protein assay (Sigma Chemicals) and SDS-PAGE analyses.

2. Nickel binding properties of recombinant proteins:

E. coli MC1061 cells, containing either the pMAL-c2 vector or derivative recombinant plasmids, were grown in 100 ml-Luria broth in the presence of carbenicillin (100 µg/ml). The expression of the genes was induced with IPTG for four hours. The cells were centrifuged and the pellet was resuspended in 2 ml of Buffer A (6M guanidine hydrochloride, 0.1 M NaH₂PO₄, 0.01 Tris, pH 8.0). After gentle stirring for one hour at room temperature, the suspensions were centrifuged at 10,000 g for 15 min at 4°C. A 1.6 ml aliquot of Nickel-Nitrilo-Tri-Acetic resin (Nickel-NTA, QIA Express), previously equilibrated in Buffer A, was added to the supernatant and this mixture was stirred at room temperature for one hour prior to loading onto a column. The column was washed with 20 ml buffer A, then 30 ml buffer B (8M urea, 0.1M Na-phosphate, 0.01M Tris-HCl, pH8.0). The proteins were eluted successively with the same buffer as buffer B adjusted to pH 6.3 (Buffer C), pH 5.9 (Buffer D) and pH 4.5 (Buffer E) and Buffer F (6M guanidine hydrochloride, 0.2 M acetic acid). Fifty µl of each fraction were mixed with 50 µl of SDS buffer and loaded on SDS gels.

3. Human sera:

Serum samples were obtained from 40 individuals, 28 were *H. pylori*-infected patients as confirmed by a positive culture for *H. pylori* and histological examination of the biopsy, and 12 were uninfected patients. The sera were kindly provided by R. J. Adamek (University of Bochum, Germany).

4. Immunoblotting:

Upon completion of SDS-PAGE runs in a Mini-PROTEAN II electrophoresis cell, proteins were transferred to nitrocellulose paper in a Mini Trans-Blot transfer cell (Bio-Rad) set at 100 V for 1 h (with cooling).

Immunostaining was performed as previously described (Ferrero et al., 1992), except that the ECL Western blotting detection system (Amersham) was used to visualize reaction products. Human sera and the rabbit antiserum, raised against a whole-cell extract of *H. pylori* strain 85P, were diluted 1:1000 and 1:5000, respectively, in 1% (w/v) casein prepared in phosphate-buffered saline (PBS, pH7.4).

5. Serological methods [enzyme-linked immunosorbent assay (ELISA)]:

The following quantities of antigens were absorbed onto 96-well plates (Falcon 3072): 2.5 µg of protein MalE, 5 µg of MalE-HspA, or 2.5 µg of MalE-HspB. The plates were left overnight at 4°C, then washed 3 times with ELISA wash solution (EWS) [1% PBS containing 0.05% (v/v) Tween 20]. Saturation was achieved by incubating the plates for 90 min at 37°C in EWS supplemented with 1% milk powder. Wells were again washed 3 times with EWS and then gently agitated for 90 min at 37°C in the presence of human sera (diluted 1:500 in EWS with 0.5% milk powder), under agitation. Bound immunoglobulins were detected by incubation for 90 min at 37°C with biotinylated secondary antibody (goat anti-human IgG, IgA or IgM diluted [1:1000] in EWS supplemented with 0.5% milk powder) in combination with streptavidin-peroxidase (1:500) (Kirkegaard and Perry Lab.). Bound peroxidase was detected by reaction with the citrate substrate and hydrogen peroxide. Plates were incubated in the dark, at room temperature, and the optical density at 492 nm was read at intervals of 5, 15 and 30 min in an ELISA plate reader. After 30 min, the reaction was stopped by the addition of hydrochloric acid to a final concentration of 0.5M.

B. Results Of Part IV Experiments:

1. Construction of recombinant plasmids producing inducible MalE-HspA, and HspB fusion proteins:

The oligonucleotides #1 and #2 (*HspA*) and #3 and #4 (*HspB*) were used to amplify by PCR the entire *HspA* and the *HspB* genes, respectively. The PCR products were electroeluted, purified and restricted with *EcoRI* and *PstI*. The restricted fragments (360 bp and 1600 bp in size, respectively) were then ligated into the *EcoRI-PstI* restricted pMAL-c2 vector to generate plasmids designated pILL933 and pILL934, respectively. Following induction with IPTG, and purification of the soluble protein on amylose columns, fusion proteins of the expected size (55 kDa for pILL933 [Figure 17], and 100 kDa for pILL934) were visualized on SDS-PAGE gels. Each of these corresponded to the fusion of the MalE protein (42.7 kDa) with the second amino acid of each of the Hsp polypeptides. The yield of the expression of the fusion proteins was 100 mg for MalE-HspA and 20 mg for MalE-HspB when prepared from 2 liters of broth culture.

2. Study of the antigenicity of the HspA and HspB fusion proteins, and of the immunogenicity of HspA and HspB in patients infected with *H. pylori*:

In order to determine whether the fusion proteins were still antigenic, each was analyzed by Western blot with rabbit antiserum raised against the MalE protein and a whole-cell extract of *H. pylori* strain 85P. Both fusion proteins were immunoreactive with antibody to MalE (not shown) and with the anti-*H. pylori* antiserum. The anti-*H. pylori* antiserum did not recognize the purified MalE protein (Fig. 18). These results demonstrated that the fusion proteins retained their antigenic properties; in addition, whereas the HspB protein was known to be immunogenic, this is the first demonstration that HspA *per se* is immunogenic in rabbits.

In the same way, in order to determine whether the HspA and HspB polypeptides were immunogenic in humans, the humoral immune response against HspA and/or HspB in patients infected with *H. pylori* was analyzed and compared to that of uninfected persons using Western immunoblotting assays and enzyme-linked immunosorbent assays (ELISA). None of the 12 sera of the *H. pylori*-negative persons gave a positive immunoblot signal with MalE, MalE-HspA, or MalE-HspB proteins (Fig. 18). In contrast, of 28 sera from *H. pylori*-positive patients, 12 (42.8%) reacted with the HspA protein whilst 20 (71.4%) recognized the HspB protein. All of the sera that recognized HspA also reacted with the HspB protein. No association was observed between the immune response and the clinical presentation of the *H. pylori* infection although such a conclusion might be premature because of the small number of strains analyzed.

3. Nickel binding properties of the fused MalE-HspA protein:

MBP-HspA recombinant protein expressed following induction with IPTG was purified from a whole cell extract by one step purification on nickel affinity column whereas the MBP alone, nor MBP-HspB exhibited this property. Figure 18 illustrates the one step purification of the MBP-HspA protein that was eluted as a monomer at pH 6.3, and as a monomer at pH 4.5. The unique band seen in panel 7 and the two bands seen in panel 5 were both specifically recognized with anti-HspA rabbit sera. This suggested that the nickel binding property of the fused MBP-HspA protein might be attributed to the C-terminal sequence of HspA, which is rich in histidine and cysteine residues.

V. Immunisation with *Helicobacter Pylori* GroES Homolog and Urease Subunit Proteins Affords Total Protection Against Mucosal Infection.

Helicobacter pylori is an etiological agent of chronic gastritis and peptic ulceration. Whilst a significant proportion of the population is infected by *H. pylori* bacteria, infected individuals do not always experience symptoms. Recent investigations have established a causal relationship between *H. pylori* and carcinogenesis, which has led to WHO/IARC to classify *H. pylori* as a "definite human carcinogen." Long-term *H. pylori* colonization of the gastric mucosa is involved in the formation of gastric atrophy, which is a known precursor of gastric cancer. It is, therefore, feasible to suggest that prophylaxis against *H. pylori* infection, as well as reducing the incidence of peptic ulcer disease, may also reduce the cases of gastric neoplasia. We believe that for such a strategy to succeed it will be necessary to target properties that are shared by all isolates of *H. pylori*.

Urease activity is a property common to all *H. pylori* isolates and is essential for colonization of the gastric mucosa. *H. pylori* urease is composed of two subunits (UreA and UreB), which form a high molecular weight complex with nickel ions. These subunits are immunodominant antigens and are highly conserved between the different gastric *Helicobacter* species, including *Helicobacter felis*.

In common with other organisms, *H. pylori* bacteria express heat-shock proteins that share homologies with the GroES and GroEL class of proteins from *Escherichia coli*. We have assessed the heat-shock proteins of *H. pylori* as potential protective antigens in a murine model of gastric *Helicobacter* infection. Orogastric immunization of mice with recombinant *H. pylori* GroES- and GroEL-like proteins protected 80% (n=20) and 70% (n=10) of animals, respectively, from a challenge dose of 10^4 *Helicobacter* bacteria (versus control mice: $P=0.0042$ and $P=0.0904$, respectively). All mice (n=19) that were immunized with a dual

antigen preparation, consisting of *H. pylori* GroES-like protein and the B subunit of *H. pylori* urease, were protected against infection. This represented an equivalent level of protection as that provided by a sonicated *Helicobacter* extract ($P=0.955$). Antibodies directed against the recombinant *H. pylori* antigens were predominantly of the IgG₁ class, suggesting a type 2 T-helper cell (Th-2) response was involved in protection.

Finally, GroES-like and urease subunit B proteins have been identified as potential components of a future *H. pylori* subunit vaccine. Presented below are data showing that the co-administration of an immunization composition of two defined antigens, *H. pylori* UreB and HspA, was able to confer a level of protection equivalent to that induced by a whole-cell preparation.

Experimental Procedures for Part V

A. Materials and Methods

1. Bacterial Strains, Media and Growth:

H. pylori (85P) was a clinical isolate. Labigne et al., J. Bacteriol, 173, 1920-1931 (1991). *H. felis* (ATCC 49179) was originally isolated from cat gastric mucosa. Lee (1988). *Helicobacters* were grown on a blood agar medium, containing an antibiotic mixture, and incubated under microaerobic conditions at 37°C. Ferrero (1993). *Escherichia coli* MC1061 cells were grown routinely at 37°C, in solid or liquid Luria medium.

2. Production of Recombinant *H. pylori* antigens:

The genes encoding *H. pylori* urease subunit B and HSP polypeptides (UreB, HspA and HspB, respectively) were each cloned into the expression vector pMAL-C2 (New England Biolabs Inc.), as previously described. Ferrero (1994) Infect. Immunol. 62, 4981-4989. Recombinant *H. pylori* proteins were expressed as MalE fusions. *E. coli* MC1061 cells harboring the recombinant

plasmids were induced with isopropyl- β -D-thiogalactopyranoside (IPTG), and the fusion proteins purified from culture supernatants by affinity and anion exchange chromatography. The purity of recombinant protein preparations was analyzed by SDS-PAGE and by immunoblotting.

3. SDS-PAGE and Immunoblotting Techniques:

Solubilized protein preparations were analyzed on slab gels, comprising a 4.5% acrylamide stacking gel and a 12.5% resolving gel, according to the procedure of Laemmli. Proteins were transferred to nitrocellulose membranes in a Mini Trans-Blot transfer cell (Bio-Rad). Immunoreactants were detected by chemiluminescence (ECL System, Amersham). Ferrero (1994).

Protein concentrations were determined by the Bradford assay (Sigma Chemical Co., St. Louis, Mo.).

4. Animal Experimentation:

Four to 6 wk-old Swiss specific-pathogen-free mice (Centre d'Elevage R. Janvier, Le-Genest-St-Isle, France) were fed a commercial pellet diet with water *ad libitum*. These mice were previously shown to be free of the murine *Helicobacter* sp, *Helicobacter muridarum* (Ferrero 1994). Aliquots (0.1 ml) containing 10^4 *H. felis* bacteria prepared from a low-subculture stock suspension of *H. felis* were administered orogastrically to mice, as previously described (Ferrero 1994). Antigen extracts (50 μ g protein) containing 5 μ g cholera toxin (Sigma) were prepared in 0.1 M sodium bicarbonate, prior to delivery to mice. Following sacrifice, stomachs were removed and sera collected.

H. felis colonization was assessed using the biopsy urease test and histological techniques. Portions of gastric antrum and body were placed on the surfaces of individual agar plates (1 cm by 1 cm) containing a modified Christensen's medium, to which had been added a *Helicobacter*-selective antibiotic mixture. The plates were observed for up to 48 h. The remaining two-thirds of each stomach were dissected into longitudinal segments

(approximate width 2 mm), which were processed for histopathology (Ferrero 1994).

So as to eliminate observer bias, Giemsa-stained sections were coded prior to histological assessment. For each stomach, all the available tissue (representing up to 2/3 of the stomach) was scrutinized. Protection from *H. felis* colonization was defined as the absence of *H. felis* bacteria from the totality of sections representing each stomach. The severity of gastritis was assessed on the basis of both the degree of mononuclear cell infiltration as well as the distribution of the cells. Thus, gastritis was scored according to the following scale: 0, no significant infiltration; 1, infiltration of low numbers of lymphocytes, limited to the muscularis mucosa and the submucosa; 2, infiltration of moderate numbers of lymphocytes in the submucosa, with variable numbers extending into the mucosa; and 3, infiltration of large numbers of lymphocytes in the mucosa, leading to the formation of several aggregates or even nodular structures.

5. ELISA:

Serum IgG antibodies in immunized mice were detected by ELISA. Sauerbaum et al., Molec. Microbiol. 14, 959-974 (1994). Briefly, 96-well plates (Nunc Maxisorb) were coated with a sonicated extract of *H. pylori* (25 µg protein per well). Bound IgG were detected with biotinylated goat anti-mouse antibodies (Amersham) and streptavidin-peroxidase conjugate. Immune complexes were detected by reaction with a solution containing o-phenylenediamine dihydrochloride (Sigma) and hydrogen peroxide. Optical density readings were read at 492 nm in an ELISA plate reader (Titertek).

6. Statistics:

Data were analyzed by χ^2 and χ^2 (with Yate's correction) tests as appropriate (Campbell et al., Medical Statistics. A Commonsense Approach, 2nd

Ed., John Wiley, Chichester (1993)), using the Statview 512⁺ computer software package (BrainPower, Inc., Calabasas, CA).

B. Results of Part V Experiments

1. Determination of the Minimum Infectious Dose for *H. felis* in the Mouse:

The *H. felis*-infected mouse has become the model of choice for trials aimed at identifying antigens that may serve in a future *H. pylori* vaccine. Thus far, the size of the *H. felis* inoculum used to challenge immunized animals has not been reconciled with the low *H. pylori* bacterial load that a vaccinated, non-infected individual would be expected to encounter when exposed to *H. pylori*-infected persons. To this end, we have determined the minimum infectious dose required to colonize Swiss mice with *H. felis* (under the conditions in our laboratory). Groups of five mice were thus colonized with inocula prepared from virulent *H. felis* bacteria, which varied from 10^1 to 10^5 bacteria. The results are shown in Table 6.

Table 6. Determination of the minimum infectious dose for *H. felis* in mice.Identification of *H. felis* infection in mice

at 2 wk post-inoculation

Inoculum dose*	Urease activity [§]	Culture [¶]
(no. of bacteria)	(no.)	(no.)
10 ¹	0/5	0/5
10 ²	4/5	3/5
10 ³	5/5	4/5
10 ⁴	5/5	3/5
10 ⁵	4/5	4/5

*To determine cell density, various dilutions of a stock *H. felis* culture (which contained predominantly helical-shaped forms) were prepared. Viable *H. felis* bacteria were then enumerated under phase contrast microscopy (magnification factor, 400 x), using a Malassez chamber. Mice were inoculated orogastrically with 0.1 ml of the appropriate inoculum containing virulent *H. felis* bacteria.

[§]Urease activity was detected in murine gastric biopsies (see *Materials and Methods*).

[¶]*H. felis* bacteria were isolated from gastric tissue biopsies after incubation on blood agar plates under microaerobic conditions for 5-7 days, at 37°C.

Whilst an inoculum containing c. 10^1 bacteria was found to be insufficient to colonize mice, gastric infection in mice was achieved with inocula containing at least 10^2 bacteria (the minimum infectious dose). A challenge inoculum equivalent to 100 times the minimum infectious dose (i.e. 10^4 bacteria) was subsequently chosen for all immunoprotection studies.

2. Protection Against *H. felis* Infection in Mice by Immunization with Recombinant HSPs from *H. pylori*.

To demonstrate the presence of HSP homologs in *H. felis*, whole-cell extracts of the organism were immunoblotted and then reacted with hyperimmune rabbit antisera raised against *H. pylori* MalE-HspA and MalE-HspB fusions. Cross-reactive antigens were detected in the *H. felis* extract: the denatured antigens had approximate molecular weights of 15 kDa and 58 kDa, respectively, which corresponded to those of the *H. pylori* HSPs (Figs. 20A, B). Interestingly, it appeared that the HspA homologs of both *H. pylori* and *H. felis* exist in dimeric forms and these multimeric forms appeared to be resistant to the denaturing effects of SDS.

Recombinant *H. pylori* HSP antigens were assessed for their potential to induce protective mucosal responses in the *H. felis* mouse model. Mice were immunized once per wk (wks 0 to 3) with 50 mg antigen (or 1 mg *H. felis* whole-cell sonicate) and 5 mg cholera toxin. At wk 5, the mice were challenged with an inoculum containing c. 10^4 *H. felis* bacteria. At wk 7, the mice were sacrificed. The results are reported in Table 7.

Table 7. Immunization of mice against *H. felis* infection using *H. pylori* antigens

Antigens	% infectious status		Grade of gastritis ^g	
	Infected (no.)	Not infected ^f	Infected	Not infected ^f
MalE (M)	14/20	30%	2.57 ± 0.65 (14)	1.0 ± 0 (6)
sonicate ^a	1/17	94	3 (1)	1.31 ± 0.79 (16)
M-HspA ^b	4/20	80	3 (4)	1.19 ± 0.83 (16)
M-HspB ^c	3/10	70	3 (3)	1.0 ± 0.82 (7)
M-UreB ^d	3/21	86	2.3 (3)	1.17 ± 0.38 (18)
M-HspA/UreB ^e	0/19	100	(h)	1.53 ± 0.70 (19)
			Σ 2.68 ± 0.56 (25) ^h	1.28 ± 0.71 (82) ⁱ

^a*P*=0.0003; ^b*P*=0.0042; ^c*P*=0.0904; ^d*P*=0.001; ^e*P*=0.0001 compared with the MalE group of animals.

^fMice were considered "not infected" when the biopsy urease test was negative, and no *H. felis* bacteria were detected in coded histological sections (see *Materials and Methods*).

^gGastitis was scored from 0 to 3 (see *Materials and Methods*). Mean scores \pm S.D. are presented. Numbers in paragraphs refer to the numbers of animals per group.

^gGastitis was scored from 0 to 3 (see *Materials and Methods*). Mean scores \pm S.D. are presented. Numbers in paragraphs refer to the numbers of animals per group.

^hNo mice from this group were infected.

ⁱComparison of score frequencies between immunized animals that became infected and those that were protected ($P=0.0001$).

^hComparison of individual scores between immunized animals that became infected and those that were protected ($P=0.0001$).

Immunization with HspA- or HspB-MalE fusions protected 80% and 70%, respectively, of mice against *H. felis* infection (Table 7). In comparison, 30% of MalE-immunized control mice did not become infected when challenged with the *H. felis* inoculum ($P=0.0042$ and $P=0.0904$, respectively).

Co-administration of recombinant *H. pylori* UreB and HspA antigens to mice resulted in 100% protection, which compared with a protection rate of 86% in those animals that had received the UreB antigen alone (Table 7). The level of protection afforded by the co-administration of MalE-UreB and MalE-HspA was equivalent to that obtained in the group of *H. felis* sonicate-immunized animals ($P=0.955$; Table 7).

3. Serological Responses Following Immunization with Recombinant HSPs and Urease Polypeptides:

Measurement of *H. pylori*-specific IgG antibodies in the serum of immunized mice demonstrated that virtually all of the animals developed strong humoral responses to the administered *H. pylori* urease and heat-shock antigens. As would be predicted of a mucosal immune response, serum antibodies directed against these antigens appeared to be primarily of the IgG₁

idiotype (Fig. 19). This finding was indicative of a predominantly type 2 T-helper cell (Th-2) response. Consistent with this, serum levels of *H. pylori*-specific IgG_{2a} antibodies, which are normally associated with Th-1 type responses, were relatively low and varied depending upon the antigen administered: HspA appeared to induce particularly weak IgG_{2a} serum responses (Fig. 19). These differences were considered to be specific to the *H. pylori* antigenic components of the recombinant proteins, since approximately equivalent levels of IgG₁ and IgG_{2a} antibody idiotypes were detected when MalE-specific antibodies were measured (unpublished data). No qualitative nor quantitative differences could be found between IgG serum responses and the infectious status of the mice at sacrifice.

4. Cellular Responses Induced in Mice following Immunization:

Histological assessment of gastric mucosa tissue from the immunized mice revealed low levels of mononuclear cells (mean inflammation score: 1.28 ± 0.71) for those mice which were protected from an *H. felis* infection (Table 7). In contrast, those immunized animals that became infected tended to have a significantly more severe form of lymphocytic gastritis in which lymphoid follicular structures were often observed (mean score: 2.68 ± 0.56 ; $P=0.0001$). Large numbers of mononuclear cells were observed in the gastric tissue of *H. felis*-colonized mice from the MalE-immunized group.

In this study, we tested an antigenic preparation consisting of two recombinant proteins, *H. pylori* UreB and HspA, and showed that, under identical experimental conditions, it was as effective as a whole-cell extract of *H. felis* in protecting against *H. felis* infection in mice. We observed in both this study, and in an independent one in which immunized mice were not challenged with *H. felis* (unpublished data), that the administration of *H. pylori* Hsp antigens did not appear to be associated with an unduly severe pathology.

The evidence to date suggests that a mild gastric inflammation may be a necessary prerequisite for a successful orogastric immunization. Michetti et al., *Gastroenterology* 107, 1002-1011 (1994); Ferrero (1994). Activation of a Th-2 immune response is normally associated with the migration of both IgA-secreting B lymphocytes and T_H lymphocytes to effector tissue sites. Staats et al., *Curr. Opin. Immunol.* 6, 572-583 (1994). It is, therefore, perhaps not surprising that orogastric immunization of mice results in a mild degree of lymphocytic gastritis. Administration of cholera toxin may contribute to this inflammation: *in vitro* experiments showed that cholera toxin alone increased the proliferation of murine B and T lymphocytes. Elson, *Infect. Immun.* 60, 2874-2879 (1992). It is also likely that the antigenic load provided by the *H. felis* bacterial challenge exacerbates the inflammation: immunized mice that became infected with *H. felis* displayed a higher degree of gastritis than those immunized animals that were protected against *H. felis* infection. However, as this difference was also observed amongst the MalE-immunized group of mice, it is unlikely that cross-reactivity between the recombinant *H. pylori* antigens and the *H. felis* bacteria accounted for the severe pathology seen in those immunized mice that were not protected. Eaton and Krakowka also observed that immunized piglets, which were not protected against *H. pylori* infection, developed severe gastritis. Eaton et al., *Gastroenterology* 103, 1580-1586 (1992).

H. pylori HspA is particularly appealing as a vaccine component because, in contrast with HspB, it possesses a unique domain at its C-terminus, which is absent from other known heat-shock homologs, including those of eucaryotic organisms. The C-terminus of *H. pylori* HspA consists of a series of 26 amino acids (out of a total of 118 amino acids), and undoubtedly confers a unique conformational structure to this polypeptide. The capacity of *H. pylori* HspA to bind to nickel ions should facilitate the large-scale purification of this polypeptide by metal affinity chromatography.

Evidence from the immunoprotection studies and immunoblot analyses suggest that *H. felis* produces a GroES homolog. Whether this protein also contains the C-terminal nickel-binding domain is currently a subject of investigation in our laboratory. It is noteworthy that these *Helicobacter* GroES homologs seem to exist as dimeric forms, a feature that has also been described for other known nickel-binding proteins, such as the UreE proteins from *Proteus mirabilis*, Sriwanthana et al., J. Bacteriol, 176, 6836-6841 (1994), and *Klebsiella aerogenes*, Lee et al., Protein Sci. 2, 1042-1052 (1993).

Thus, the immunization composition of this invention preferably contains *H. pylori* UreB and HspA as immunogens. The UreB and HspA can be isolated from *H. pylori* lysates or sonicates, but are preferably free of other *H. pylori* antigens, including multimeric urease. Thus, in one embodiment of the invention the UreB and HspA are substantially free of UreA. It is particularly preferred that the UreB and the HspA be prepared by recombinant techniques. The resulting recombinant antigens are substantially free of multimeric urease and other *H. pylori* antigens.

The immunization composition of the invention can also include an adjuvant in an amount sufficient to enhance the magnitude or duration of the immune response in the host, or to enhance the qualitative response in the subject, such as by stimulating antibodies of different immunoglobulin classes than those stimulated by the immunogen. The adjuvant should efficiently elicit cell-mediated or humoral immune responses to antigens without systemic or localized irritation of the host system. Preferably, the adjuvant has low pyrogenicity.

Well known adjuvant formulations for human or veterinary applications can be employed. Such adjuvants can be based on emulsions, with or without mycobacteria, or adjuvants based on adsorption of antigens to aluminum salts, especially aluminum hydroxide or aluminum phosphate. Among these adjuvants

are oil adjuvants based on mineral, animal, and vegetable oils. Oil based adjuvants are useful for increasing humoral responses of animals to vaccine antigens, and certain oil-based adjuvants have been tested for human use. Typical adjuvants are Freund's complete adjuvant and Freund's incomplete adjuvant.

Suitable adjuvants that have been developed more recently, include liposomes, immune-stimulating complexes (ISCOMs), and squalene or squalene emulsions. Surface active agents having adjuvant activity can also be employed. These include saponin-like Quil A molecules in ISCOMs and Pluronic® block copolymers that are used to make stable squalene emulsions. Saponins are surface-active agents widely distributed in plants.

Analogues of muramyl dipeptide (MDP) or muramyl tripeptide (MTP), such as threonine analogue of MDP and lipopolysaccharide (LPS) having adjuvant activity and reduced side effects, are also suitable for use as adjuvants. Synthetic analogues of MDP and the monophosphoryl derivative of lipid A are also known for their adjuvant activity and reduced pyrogenicity. A particularly suitable formulation is Syntex Adjuvant Formulation-1 or SAF-1, which combines the threonyl analogue of MDP in a vehicle comprised of Pluronic L-121 triblock polymer with squalene and a small proportion of Tween 80 as an emulsifying detergent. The preferred adjuvants for use in humans are MDP and its analogues, with or without squalene, saponins, and the monophosphoryl derivative of lipid A. When an adjuvant is combined with the immunogen in the composition and method of the invention, a further enhancement in immune response is observed.

A preferred route of administering the composition of the invention to a host is mucosal. Oral administration is the particularly preferred mode of administration because of its simplicity and because it is relatively non-invasive. It will be understood that the immunogenic composition of the invention can also

be employed in a vaccine. An alternative mucosal adjuvant could be used. All or part of the cholera (CT) or *E. coli* LT holotoxins in either toxic or detoxified forms are examples.

The composition of the invention can be incorporated into any suitable delivery system. For example, the antigen and adjuvant can be combined with a pharmaceutically acceptable liquid vehicle, such as water, buffered saline, or edible animal or vegetable oil. The composition can be combined with one or more suitable pharmaceutically acceptable excipients or core materials, such as cellulose, cellulose derivatives, sucrose, gelatin, Starch 1500, NuTab, lactose, malto-dextrin, talc, Cabosil, magnesium stearate, alginate, Actisol, PEG 400, Myvacet, Triacetine, syrup, oil, sorbitol, mannitol, and Plasdane. This list is not intended to be exhaustive or limiting; alternative or additional excipients or core materials can also be used.

It will also be understood that the compositions of the invention can be formulated to include chemical agents that are capable of neutralizing stomach pH. Suitable neutralizing agents include H_2 antagonists, proton pump inhibitors, bicarbonate of soda, calcium carbonate, and aluminum hydroxide.

The composition of the invention can be utilized in the form of elixirs, solutions, suspensions, syrups, aerosols, and the like. The composition can also be prepared in dosage units suitable for oral or parenteral administration, such as particles, granules, beads, tablets, hard gelatin capsules, and soft gelatin capsules.

The immunogen and adjuvant are employed in a combined amount to provide an immune response against an infectious agent. This can be determined by estimating seroconversion, that is, the levels of antibody before and after immunization. If the host has a preexisting antibody titer to the antigen, the success of immunization can be determined by the extent of increase in the level of specific antibody. In cases where there is no correlation between

seroconversion and protection, cell-mediated immune response can be monitored.

The amount of antigen and adjuvant per dosage unit will depend on the desired dose and the frequency of administration. In one embodiment, each dosage unit contains an amount of antigen effective to protect the animal against disease following exposure to the pathogen. The dose can be defined as the amount of immunogen necessary to raise an immune response against *H. pylori* infection in an individual. As an example, the immunization schedule in animals (mice) consists of 4 administrations (one/week). Each oral dose unit (one per week) comprises 250 to 900 micrograms of UreB and 250 to 900 micrograms of HspA and 25 to 90 micrograms of adjuvant. A suitable weight ratio of UreB:HspA:adjuvant is 1:1:0.1, but it will be understood that other ratios of ingredients can be employed. The average weight of a mouse is 20 g and one can calculate for one kilogram of other animal or a human patient to be immunized the equivalent dose unit. The precise composition will necessarily vary depending on the antigen and adjuvant selected, the species to be immunized, and other factors, and it is within the capacity of one with ordinary skill in the art to search for an optimal formulation.

The immunogenic composition can be administered before or after infection. A booster dose can comprise the antigen in an amount sufficient to enhance the initial immune response. It has to be adapted to each protocol depending on the antigen and the host. Multiple doses may be more appropriate for children and for individuals with no known prior exposure.

The immunogenic composition containing UreB and HspA can be administered to an infected or non-infected animal. Thus, it will be understood that this invention can be employed for the prophylactic, therapeutic, or curative treatment of any animal in need thereof, such as dogs, cats, poultry, pigs,

horses, and cattle, and especially mammals, such as primates, including humans, using UreB and HspA or the species equivalent thereof.

Finally, a preferred embodiment of the previously described antibodies of the invention comprises monoclonal antibodies, polyclonal antibodies, or fragments of such antibodies that immunologically recognize UreB, HspA, or mixtures of UreB and HspA. Antibodies and antibody fragments that are specific for these polypeptides and their immunologically recognizable fragments can be prepared by the techniques described above.

Inasmuch as the present invention is subject to many variations, modifications, and changes in details, it is intended that all subject matter discussed above or shown in the accompanying drawings be interpreted as illustrative and not in a limiting sense. Such modifications and variations are included within the scope of this invention as defined by the following claims.

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Turbett, G. R., Hoj, P., Horne, R., and Mee, B. J. (1992) Purification and characterization of the urease enzymes of Helicobacter species from humans and animals. Infect Immun 60: 5259-5266.

95.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: INSTITUT PASTEUR
(B) STREET: 25-28 rue du Dr Roux
(C) CITY: PARIS CEDEX 15
(E) COUNTRY: FRANCE
(F) POSTAL CODE (ZIP): 75724
(G) TELEPHONE: 45.68.80.94
(H) TELEFAX: 40.61.30.17

(A) NAME: INSTITUT NATIONAL DE LA SANTE ET DE LA
RECHERCHE MEDICALE
(B) STREET: 101 rue de Tolbiac
(C) CITY: PARIS CEDEX 13
(E) COUNTRY: FRANCE
(F) POSTAL CODE (ZIP): 75654
(G) TELEPHONE: 44.23.60.00
(H) TELEFAX: 45.85.07.66

(ii) TITLE OF INVENTION: IMMUNOGENIC COMPOSITIONS AGAINST HELICOBACTER
INFECTION, POLYPEPTIDES FOR USE IN THE COMPOSITIONS AND
NUCLEIC ACID SEQUENCES ENCODING SAID POLYPEPTIDES.

(iii) NUMBER OF SEQUENCES: 19

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: PCT/EP 94/01625

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: EP 93401309.5
(B) FILING DATE: 19-MAY-1993

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: PCT/EP 93/03259
(B) FILING DATE: 19-NOV-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2619 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
(B) LOCATION: 31..36
(D) OTHER INFORMATION: /standard_name= "Shine-Dalgarno
sequence"

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
(B) LOCATION: 756..759
(D) OTHER INFORMATION: /standard_name= "Shine-Dalgarno
sequence"

(ix) FEATURE:

- (A) NAME/KEY: CDS
(B) LOCATION: 43..753
(D) OTHER INFORMATION: /standard_name= "URE A - FIGURE 3"

(ix) FEATURE:

- (A) NAME/KEY: CDS
(B) LOCATION: 766..2475
(D) OTHER INFORMATION: /standard name= "URE B - FIGURE 3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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<p> GAA GAA CGC TTG GCG CGT GGT GTG AAA CTC AAT TAC ACC GAA GCG GTC Glu Glu Arg Leu Ala Arg Gly Val Lys Leu Asn Tyr Thr Glu Ala Val 25 30 35 </p>	150
<p> GCG CTC ATT AGC GGG CGT GTG ATG GAA AAG GCG CGT GAT GGT AAT AAA Ala Leu Ile Ser Gly Arg Val Met Glu Lys Ala Arg Asp Gly Asn Lys 40 45 50 </p>	198
<p> AGC GTG GCG GAT TTG ATG CAA GAA GGC AGG ACT TGG CTT AAA AAA GAA Ser Val Ala Asp Leu Met Gln Glu Gly Arg Thr Trp Leu Lys Lys Glu 55 60 65 </p>	246

AAT GTG ATG GAC GGC GTA GCA AGC ATG ATT CAT GAA GTG GGG ATT GAA Asn Val Met Asp Gly Val Ala Ser Met Ile His Glu Val Gly Ile Glu 70 75 80	294
GCT AAC TTC CCC GAT GGA ACC AAG CTT GTA ACT ATC CAC ACT CCG GTA Ala Asn Phe Pro Asp Gly Thr Lys Leu Val Thr Ile His Thr Pro Val 85 90 95 100	342
GAG GAT AAT GGC AAA TTA GCC CCC GGC GAG GTC TTC TTA AAA AAT GAG Glu Asp Asn Gly Lys Leu Ala Pro Gly Glu Val Phe Leu Lys Asn Glu 105 110 115	390
GAC ATT ACT ATT AAC GCC GGC AAA GAA GCC ATT AGC TTG AAA GTG AAA Asp Ile Thr Ile Asn Ala Gly Lys Glu Ala Ile Ser Leu Lys Val Lys 120 125 130	438
AAT AAA GGC GAT CGT CCT GTG CAG GTG GGA TCA CAT TTC CAC TTC TTC Asn Lys Gly Asp Arg Pro Val Gln Val Gly Ser His Phe His Phe Phe 135 140 145	486
GAA GTG AAT AAG CTC TTG GAC TTC GAT CGC GCA AAA AGC TTT TGC AAA Glu Val Asn Lys Leu Leu Asp Phe Asp Arg Ala Lys Ser Phe Cys Lys 150 155 160	534
CGC CTA GAC ATT GCA TCT GGA ACA GCG GTG CGC TTT GAA CCC GGG GAG Arg Leu Asp Ile Ala Ser Gly Thr Ala Val Arg Phe Glu Pro Gly Glu 165 170 175 180	582
GAA AAA AGT GTG GAA CTC ATT GAC ATC GGC GGG AAT AAG CGC ATC TAT Glu Lys Ser Val Glu Leu Ile Asp Ile Gly Gly Asn Lys Arg Ile Tyr 185 190 195	630
GGC TTT AAT TCT TTG GTG GAT CGC CAA GCC GAT GCC GAT GGT AAA AAA Gly Phe Asn Ser Leu Val Asp Arg Gln Ala Asp Ala Asp Gly Lys Lys 200 205 210	678
CTC GGC TTA AAA CGC GCT AAA GAA AAA GGT TTT GGG TCT GTA AAC TGC Leu Gly Leu Lys Arg Ala Lys Glu Lys Gly Phe Gly Ser Val Asn Cys 215 220 225	726
GGT TGT GAA GCG ACT AAA GAT AAA CAA TAAGGAAAAA CC ATG AAA AAG Gly Cys Glu Ala Thr Lys Asp Lys Gln Met Lys Lys 230 235 1	774
ATT TCA CGA AAA GAA TAT GTT TCT ATG TAT GGT CCC ACT ACC GGG GAT Ile Ser Arg Lys Glu Tyr Val Ser Met Tyr Gly Pro Thr Thr Gly Asp 5 10 15	822
CGT GTT AGA CTC GGC GAC ACT GAT TTG ATC TTA GAA GTG GAG CAT GAT Arg Val Arg Leu Gly Asp Thr Asp Leu Ile Leu Glu Val Glu His Asp 20 25 30 35	870
TGC ACC ACT TAT GGT GAA GAG ATC AAA TTT GGG GGC GGT AAA ACT ATC Cys Thr Thr Tyr Gly Glu Glu Ile Lys Phe Gly Gly Gly Lys Thr Ile 40 45 50	918

CGT GAT GGG ATG AGT CAA ACC AAT AGC CCT AGC TCT TAT GAA TTA GAT	966
Arg Asp Gly Met Ser Gln Thr Asn Ser Pro Ser Ser Tyr Glu Leu Asp	
55 60 65	
TTG GTG CTC ACT AAC GCC CTC ATT GTG GAC TAT ACG GGC ATT TAC AAA	1014
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70 75 80	
GCC GAC ATT GGG ATT AAA GAC GGC AAG ATT GCA GGC ATT GGC AAG GCA	1062
Ala Asp Ile Gly Ile Lys Asp Gly Lys Ile Ala Gly Ile Gly Lys Ala	
85 90 95	
GGC AAT AAG GAC ATG CAA GAT GGC GTA GAT AAT AAT CTT TGC GTA GGT	1110
Gly Asn Lys Asp Met Gln Asp Gly Val Asp Asn Asn Leu Cys Val Gly	
100 105 110 115	
CCT GCT ACA GAG GCT TTG GCA GCT GAG GGC TTG ATT GTA ACC GCT GGT	1158
Pro Ala Thr Glu Ala Leu Ala Ala Glu Gly Leu Ile Val Thr Ala Gly	
120 125 130	
GGC ATC GAT ACG CAT ATT CAC TTT ATC TCT CCC CAA CAA ATC CCT ACT	1206
Gly Ile Asp Thr His Ile His Phe Ile Ser Pro Gln Gln Ile Pro Thr	
135 140 145	
GCT TTT GCC AGC GGG GTT ACA ACC ATG ATT GGA GGA GGC ACA GGA CCT	1254
Ala Phe Ala Ser Gly Val Thr Thr Met Ile Gly Gly Gly Thr Gly Pro	
150 155 160	
GCG GAT GGC ACG AAT GCG ACC ACC ATC ACT CCC GGA CGC GCT AAT CTA	1302
Ala Asp Gly Thr Asn Ala Thr Thr Ile Thr Pro Gly Arg Ala Asn Leu	
165 170 175	
AAA AGT ATG TTG CGT GCA GCC GAA GAA TAC GCC ATG AAT CTA GGC TTT	1350
Lys Ser Met Leu Arg Ala Ala Glu Glu Tyr Ala Met Asn Leu Gly Phe	
180 185 190 195	
TTG GCT AAG GGG AAT GTG TCT TAC GAA CCC TCT TTA CGC GAT CAG ATT	1398
Leu Ala Lys Gly Asn Val Ser Tyr Glu Pro Ser Leu Arg Asp Gln Ile	
200 205 210	
GAA GCA GGG GCG ATT GGT TTT AAA ATC CAC GAA GAC TGG GGA AGC ACA	1446
Glu Ala Gly Ala Ile Gly Phe Lys Ile His Glu Asp Trp Gly Ser Thr	
215 220 225	
CCT GCA GCT ATT CAC CAC TGC CTC AAT GTC GCC GAT GAA TAC GAT GTG	1494
Pro Ala Ala Ile His His Cys Leu Asn Val Ala Asp Glu Tyr Asp Val	
230 235 240	
CAA GTG GCT ATC CAC ACC GAT ACC CTT AAC GAG GCG GGC TGT GTA GAA	1542
Gln Val Ala Ile His Thr Asp Thr Leu Asn Glu Ala Gly Cys Val Glu	
245 250 255	
GAC ACC CTA GAG GCG ATT GCC GGG CGC ACC ATC CAT ACC TTC CAC ACT	1590
Asp Thr Leu Glu Ala Ile Ala Gly Arg Thr Ile His Thr Phe His Thr	
260 265 270 275	

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GAA GGG GCT GGG GGT GGA CAC GCT CCA GAT GTT ATC AAA ATG GCA GGG Glu Gly Ala Gly Gly Gly His Ala Pro Asp Val Ile Lys Met Ala Gly 280 285 290	1638
GAA TTT AAC ATT CTA CCC GCC TCT ACT AAC CCG ACC ATT CCT TTC ACC Glu Phe Asn Ile Leu Pro Ala Ser Thr Asn Pro Thr Ile Pro Phe Thr 295 300 305	1686
AAA AAC ACT GAA GCC GAG CAC ATG GAC ATG TTA ATG GTG TGC CAC CAC Lys Asn Thr Glu Ala Glu His Met Asp Met Leu Met Val Cys His His 310 315 320	1734
TTG GAT AAA AGT ATC AAG GAA GAT GTG CAG TTT GCC GAT TCG AGG ATT Leu Asp Lys Ser Ile Lys Glu Asp Val Gln Phe Ala Asp Ser Arg Ile 325 330 335	1782
CGC CCC CAA ACT ATC GCG GCT GAA GAC CAA CTC CAT GAC ATG GGG ATC Arg Pro Gln Thr Ile Ala Ala Glu Asp Gln Leu His Asp Met Gly Ile 340 345 350 355	1830
TTT TCT ATC ACC AGC TCC GAC TCT CAG GCT ATG GGA CGC GTA GGC GAG Phe Ser Ile Thr Ser Ser Asp Ser Gln Ala Met Gly Arg Val Gly Glu 360 365 370	1878
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CGC TAC ATC TCT AAA TAC ACC ATC AAC CCC GGG ATC GCG CAT GGG ATT Arg Tyr Ile Ser Lys Tyr Thr Ile Asn Pro Gly Ile Ala His Gly Ile 405 410 415	2022
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CTT TGG AGT CCG GCT TTC TTT GGC ATT AAG CCC AAT ATG ATT ATT AAG Leu Trp Ser Pro Ala Phe Phe Gly Ile Lys Pro Asn Met Ile Ile Lys 440 445 450	2118
GGC GGA TTT ATT GCG CTC TCT CAA ATG GGC GAT GCC AAT GCG TCT ATT Gly Gly Phe Ile Ala Leu Ser Gln Met Gly Asp Ala Asn Ala Ser Ile 455 460 465	2166
CCC ACC CCT CAG CCC GTC TAT TAC CGT GAA ATG TTT GGA CAC CAT GGG Pro Thr Pro Gln Pro Val Tyr Tyr Arg Glu Met Phe Gly His His Gly 470 475 480	2214
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GTG AAA AAC TGT CGC AAT ATC ACT AAA AAG GAC CTC AAA TTC AAC GAT	2358
Val Lys Asn Cys Arg Asn Ile Thr Lys Lys Asp Leu Lys Phe Asn Asp	
520 525 530	
GTG ACC GCA CAT ATT GAT GTC AAC CCT GAA ACC TAT AAG GTG AAA GTG	2406
Val Thr Ala His Ile Asp Val Asn Pro Glu Thr Tyr Lys Val Lys Val	
535 540 545	
GAT GGC AAA GAG GTA ACC TCT AAA GCA GCA GAT GAA TTG AGC CTA GCG	2454
Asp Gly Lys Glu Val Thr Ser Lys Ala Ala Asp Glu Leu Ser Leu Ala	
550 555 560	
CAA CTT TAT AAT TTG TTC AGGAGGCTA AGGAGGGGGA TAGAGGGGGT	2502
Gln Leu Tyr Asn Leu Phe	
565 570	
TTATTTAGAG GGGAGTCATT GATTTACCTT TGCTAGTTTA TAATGGATTT AAGAGAGGTT	2562
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 237 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Helicobacter felis

(ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..237
- (D) OTHER INFORMATION: /note= "Corresponds to figure 4, line 1 (ure A)."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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1				5					10					15	
Gly	Arg	Leu	Ala	Glu	Glu	Arg	Leu	Ala	Arg	Gly	Val	Lys	Leu	Asn	Tyr
		20					25						30		
Thr	Glu	Ala	Val	Ala	Leu	Ile	Ser	Gly	Arg	Val	Met	Glu	Lys	Ala	Arg
		35					40					45			

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Asp Gly Asn Lys Ser Val Ala Asp Leu Met Gln Glu Gly Arg Thr Trp
 50                               55                               60

Leu Lys Lys Glu Asn Val Met Asp Gly Val Ala Ser Met Ile His Glu
 65                               70                               75                               80

Val Gly Ile Glu Ala Asn Phe Pro Asp Gly Thr Lys Leu Val Thr Ile
                               85                               90                               95

His Thr Pro Val Glu Asp Asn Gly Lys Leu Ala Pro Gly Glu Val Phe
          100                               105                               110

Leu Lys Asn Glu Asp Ile Thr Ile Asn Ala Gly Lys Glu Ala Ile Ser
          115                               120                               125

Leu Lys Val Lys Asn Lys Gly Asp Arg Pro Val Gln Val Gly Ser His
          130                               135                               140

Phe His Phe Phe Glu Val Asn Lys Leu Leu Asp Phe Asp Arg Ala Lys
          145                               150                               155                               160

Ser Phe Cys Lys Arg Leu Asp Ile Ala Ser Gly Thr Ala Val Arg Phe
          165                               170                               175

Glu Pro Gly Glu Glu Lys Ser Val Glu Leu Ile Asp Ile Gly Gly Asn
          180                               185                               190

Lys Arg Ile Tyr Gly Phe Asn Ser Leu Val Asp Arg Gln Ala Asp Ala
          195                               200                               205

Asp Gly Lys Lys Leu Gly Leu Lys Arg Ala Lys Glu Lys Gly Phe Gly
          210                               215                               220

Ser Val Asn Cys Gly Cys Glu Ala Thr Lys Asp Lys Gln
          225                               230                               235

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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 569 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Helicobacter felis*

(ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..569
- (D) OTHER INFORMATION: /note= "Corresponds to figure 4,
line 1 (ure B)."

SUBSTITUTE SHEET (RULE 26)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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Thr Gly Asp Arg Val Arg Leu Asp Leu Ile Leu Glu Val
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Glu His Asp Cys Thr Thr Tyr Gly Gln Lys Phe Gly Gly Gly
 35 40 45

Thr Ile Arg Asp Gly Met Ser Gln Thr Pro Ser Ser Tyr
 50 55

Asp Leu Val Leu Thr Asn Ala Leu Ile Val Tyr Thr Gly
 60 70 75 80

Ile Ala Asp Ile Gly Ile Lys Asp Gly Lys Ile Ala Gly Ile
 85 90 95

Gly Lys Ala Gly Asn Lys Asp Met Gln Asp Gly Val Asp Asn Asn Leu
 100 105 110

Cys Val Gly Pro Ala Thr Glu Ala Leu Ala Ala Glu Gly Leu Ile Val
 115 120 125

Thr Ala Gly Gly Ile Asp Thr His Ile His Phe Ile Ser Pro Gln Gln
 130 135 140

Ile Pro Thr Ala Phe Ala Ser Gly Val Thr Thr Met Ile Gly Gly Gly
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Thr Gly Pro Ala Asp Gly Thr Asn Ala Thr Thr Ile Thr Pro Gly Arg
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Ala Asn Leu Lys Ser Met Leu Arg Ala Ala Glu Glu Tyr Ala Met Asn
 180 185 190

Leu Gly Phe Leu Ala Lys Gly Asn Val Ser Tyr Glu Pro Ser Leu Arg
 195 200 205

Asp Gln Ile Glu Ala Gly Ala Ile Gly Phe Lys Ile His Glu Asp Trp
 210 215 220

Gly Ser Thr Pro Ala Ala Ile His His Cys Leu Asn Val Ala Asp Glu
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Tyr Asp Val Gln Val Ala Ile His Thr Asp Thr Leu Asn Glu Ala Gly
 245 250 255

Cys Val Glu Asp Thr Leu Glu Ala Ile Ala Gly Arg Thr Ile His Thr
 260 265 270

Phe His Thr Glu Gly Ala Gly Gly Gly His Ala Pro Asp Val Ile Lys
 275 280 285

Met Ala Gly Glu Phe Asn Ile Leu Pro Ala Ser Thr Asn Pro Thr Ile
 290 295 300

SUBSTITUTE SHEET (RULE 26)

Pro Phe Thr Lys Asn Thr Glu Ala Glu His Met Asp Met Leu Met Val
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 Cys His His Leu Asp Lys Ser Ile Lys Glu Asp Val Gln Phe Ala Asp
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 Ser Arg Ile Arg Pro Gln Thr Ile Ala Ala Glu Asp Gln Leu His Asp
 340 345 350
 Met Gly Ile Phe Ser Ile Thr Ser Ser Asp Ser Gln Ala Met Gly Arg
 355 360 365
 Val Gly Glu Val Ile Thr Arg Thr Trp Gln Thr Ala Asp Lys Asn Lys
 370 375 380
 Lys Glu Phe Gly Arg Leu Lys Glu Glu Lys Gly Asp Asn Asp Asn Phe
 385 390 395 400
 Arg Ile Lys Arg Tyr Ile Ser Lys Tyr Thr Ile Asn Pro Gly Ile Ala
 405 410 415
 His Gly Ile Ser Asp Tyr Val Gly Ser Val Glu Val Gly Lys Tyr Ala
 420 425 430
 Asp Leu Val Leu Trp Ser Pro Ala Phe Phe Gly Ile Lys Pro Asn Met
 435 440 445
 Ile Ile Lys Gly Gly Phe Ile Ala Leu Ser Gln Met Gly Asp Ala Asn
 450 455 460
 Ala Ser Ile Pro Thr Pro Gln Pro Val Tyr Tyr Arg Glu Met Phe Gly
 465 470 475 480
 His His Gly Lys Asn Lys Phe Asp Thr Asn Ile Thr Phe Val Ser Gln
 485 490 495
 Ala Ala Tyr Lys Ala Gly Ile Lys Glu Glu Leu Gly Leu Asp Arg Ala
 500 505 510
 Ala Pro Pro Val Lys Asn Cys Arg Asn Ile Thr Lys Lys Asp Leu Lys
 515 520 525
 Phe Asn Asp Val Thr Ala His Ile Asp Val Asn Pro Glu Thr Tyr Lys
 530 535 540
 Val Lys Val Asp Gly Lys Glu Val Thr Ser Lys Ala Ala Asp Glu Leu
 545 550 555 560
 Ser Leu Ala Gln Leu Tyr Asn Leu Phe
 565

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2284 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME: CDS
- (B) LOCATION: 124..477
- (D) OTHER INFORMATION: /standard_name= "H. pylori - Hsp A"

(ix) FEATURE:

- (A) NAME: CDS
- (B) LOCATION: 806..2143
- (D) OTHER INFORMATION: /standard_name= "H. pylori - Hsp B"

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..2284
- (D) OTHER INFORMATION: /note= "Corresponds to figure 6."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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TGTCGCTTAA GAATACTAAG CGCTAAATTT CTATTTTATT TATCAAAACT TAGGAGAACT      120
GAA ATG AAG TTT CAA CCA TTA GGA GAA AGG GTC TTA GTA GAA AGA CTT      168
  Met Lys Phe Gln Pro Leu Gly Glu Arg Val Leu Val Glu Arg Leu
    1             5             10             15
GAA GAA GAG AAC AAA ACC AGT TCA GGC ATC ATC ATC CCT GAT AAC GCT      216
Glu Glu Glu Asn Lys Thr Ser Ser Gly Ile Ile Ile Pro Asp Asn Ala
    20             25             30
AAA GAA AAG CCT TTA ATG GGC GTA GTC AAA GCG GTT AGC CAT AAA ATC      264
Lys Glu Lys Pro Leu Met Gly Val Val Lys Ala Val Ser His Lys Ile
    35             40             45
AGT GAG GGT TGC AAA TGC GTT AAA GAA GGC GAT GTG ATC GCT TTT GGC      312
Ser Glu Gly Cys Lys Cys Val Lys Glu Gly Asp Val Ile Ala Phe Gly
    50             55             60
AAA TAC AAA GGC GCA GAA ATC GTT TTA GAT GGC GTT GAA TAC ATG GTG      360
Lys Tyr Lys Lys Gly Ala Glu Ile Val Leu Asp Gly Val Glu Tyr Met Val
    65             70             75
CTA GAA CTA GAA GAC ATT CTA GGT ATT GTG GGC TCA GGC TCT TGC TGT      408
Leu Glu Leu Glu Asp Ile Leu Gly Ile Val Gly Ser Gly Ser Cys Cys
    80             85             90             95

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SUBSTITUTE SHEET (RULE 26)

CAT ACA GGT AAT CAT GAT CAT AAA CAT GCT AAA GAG CAT GAA GCT TGC	456
His Thr Gly Asn His Asp His Lys His Ala Lys Glu His Glu Ala Cys	
100 105 110	
TGT CAT GAT CAC AAA AAA CAC TAAAAAACAT TATTATTAAG GATACAAA ATG	508
Cys His Asp His Lys Lys His Met	
115 1	
GCA AAA GAA ATC AAA TTT TCA GAT AGC GCA AGA AAC CTT TTA TTT GAA	556
Ala Lys Glu Ile Lys Phe Ser Asp Ser Ala Arg Asn Leu Leu Phe Glu	
5 10 15	
GGC GTA AGA CAA CTC CAT GAC GCT GTC AAA GTA ACC ATG GGG CCA AGA	604
Gly Val Arg Gln Leu His Asp Ala Val Lys Val Thr Met Gly Pro Arg	
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Gly Arg Asn Val Leu Ile Gln Lys Ser Tyr Gly Ala Pro Ser Ile Thr	
35 40 45	
AAA GAC GGC GTG AGC GTG GCT AAA GAG ATT GAA TTA AGT TGC CCC GTG	700
Lys Asp Gly Val Ser Val Ala Lys Glu Ile Glu Leu Ser Cys Pro Val	
50 55 60 65	
GCT AAC ATG GGC GCT CAG CTC GTT AAA GAA GAT GCG AGC AAA ACC GCT	748
Ala Asn Met Gly Ala Gln Leu Val Lys Glu Asp Ala Ser Lys Thr Ala	
70 75 80	
GAT GCC GCC GGC GAT GGC ACG ACC ACA GCG ACC GTG CTG GCT TAT AGC	796
Asp Ala Ala Gly Asp Gly Thr Thr Thr Ala Thr Val Leu Ala Tyr Ser	
85 90 95	
ATT TTT AAA GAG GGC TTG AGG AAT ATC ACG GCT GGG GCT AAC CCT ATT	844
Ile Phe Lys Glu Gly Leu Arg Asn Ile Thr Ala Gly Ala Asn Pro Ile	
100 105 110	
GAA GTG AAA CGA GGC ATG GAT AAA GCG CCT GAA GCG ATC ATT AAT GAG	892
Glu Val Lys Arg Gly Met Asp Lys Ala Pro Glu Ala Ile Ile Asn Glu	
115 120 125	
CTT AAA AAA GCG AGC AAA AAA GTG GGC GGT AAA GAA GAA ATC ACC CAA	940
Leu Lys Lys Ala Ser Lys Lys Val Gly Gly Lys Glu Glu Ile Thr Gln	
130 135 140 145	
GTA GCG ACC ATT TCT GCA AAC TCC GAT CAC AAT ATC GGG AAA CTC ATC	988
Val Ala Thr Ile Ser Ala Asn Ser Asp His Asn Ile Gly Lys Leu Ile	
150 155 160	
GCT GAC GCT ATG GAA AAA GTG GGT AAA GAC GGC GTG ATC ACC GTT GAA	1036
Ala Asp Ala Met Glu Lys Val Gly Lys Asp Gly Val Ile Thr Val Glu	
165 170 175	
GAA GCT AAG GGC ATT GAA GAT GAA TTA GAT GTC GTA GAA GGC ATG CAA	1084
Glu Ala Lys Gly Ile Glu Asp Glu Leu Asp Val Val Glu Gly Met Gln	
180 185 190	

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TTT GAT AGA GGC TAC CTC TCC CCT TAC TTT GTA ACC AAC GCT GAG AAA	1132
Phe Asp Arg Gly Tyr Leu Ser Pro Tyr Phe Val Thr Asn Ala Glu Lys	
195 200 205	
ATG ACC GCT CAA TTG GAT AAC GCT TAC ATC CTT TTA ACG GAT AAA AAA	1180
Met Thr Ala Gln Leu Asp Asn Ala Tyr Ile Leu Leu Thr Asp Lys Lys	
215 220 225	
TC ATG AAA GAC ATT CTC CCG CTA CTA GAA AAA ACC ATG AAA	1228
Ile Met Lys Asp Ile Leu Pro Leu Leu Glu Lys Thr Met Lys	
230 235 240	
GAG GGC TTT TTA ATC ATC GCT GAA GAC ATT GAG GGC GAA GCT	1276
Glu Gly Lys Leu Ile Ile Ala Glu Asp Ile Glu Gly Glu Ala	
250 255	
TTA ACG ACT CTA GTG GTG AAT AAA TTA AGA GGC GTG TTG AAT ATC GCA	1324
Leu Thr Thr Leu Val Val Asn Lys Leu Arg Gly Val Leu Asn Ile Ala	
260 265 270	
GCG GTT AAA GCT CCA GGC TTT GGG GAC AGG AGA AAA GAA ATG CTC AAA	1372
Ala Val Lys Ala Pro Gly Phe Gly Asp Arg Arg Lys Glu Met Leu Lys	
275 280 285	
GAC ATC GCT GTT TTA ACC GGC GGT CAA GTC ATT AGC GAA GAA TTG GGC	1420
Asp Ile Ala Val Leu Thr Gly Gly Gln Val Ile Ser Glu Glu Leu Gly	
290 295 300 305	
TTG AGT CTA GAA AAC GCT GAA GTG GAG TTT TTA GGC AAA GCG AAG ATT	1468
Leu Ser Leu Glu Asn Ala Glu Val Glu Phe Leu Gly Lys Ala Lys Ile	
310 315 320	
GTG ATT GAC AAA GAC AAC ACC ACG ATC GTA GAT GGC AAA GGC CAT AGC	1516
Val Ile Asp Lys Asp Asn Thr Thr Ile Val Asp Gly Lys Gly His Ser	
325 330 335	
CAT GAC GTC AAA GAC AGA GTC GCG CAA ATC AAA ACC CAA ATT GCA AGC	1564
His Asp Val Lys Asp Arg Val Ala Gln Ile Lys Thr Gln Ile Ala Ser	
340 345 350	
ACG ACA AGC GAT TAC GAC AAA GAA AAA TTG CAA GAA AGA TTG GCC AAA	1612
Thr Thr Ser Asp Tyr Asp Lys Glu Lys Leu Gln Glu Arg Leu Ala Lys	
355 360 365	
CTC TCT GGC GGT GTG GCT GTG ATT AAA GTG GGC GCT GCG AGT GAA GTG	1660
Leu Ser Gly Gly Val Ala Val Ile Lys Val Gly Ala Ala Ser Glu Val	
370 375 380 385	
GAA ATG AAA GAG AAA AAA GAC CGG GTG GAT GAC GCG TTG AGC GCG ACT	1708
Glu Met Lys Glu Lys Lys Asp Arg Val Asp Asp Ala Leu Ser Ala Thr	
390 395 400	
AAA GCG GCG GTT GAA GAA GGC ATT GTG ATT GGG GGC GGT GCG GCC CTC	1756
Lys Ala Ala Val Glu Glu Gly Ile Val Ile Gly Gly Gly Ala Ala Leu	
405 410 415	

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ATT CGC GCG GCC CAA AAA GTG CAT TTG AAT TTA CAC GAT GAT GAA AAA	1804
Ile Arg Ala Ala Gln Lys Val His Leu Asn Leu His Asp Asp Glu Lys	
420 425 430	
GTG GGC TAT GAA ATC ATC ATG CGC GCC ATT AAA GCC CCA TTA GCT CAA	1852
Val Gly Tyr Glu Ile Ile Met Arg Ala Ile Lys Ala Pro Leu Ala Gln	
435 440 445	
ATC GCT ATC AAT GCC GGT TAT GAT GGC GGT GTG GTC GTG AAT GAA GTA	1900
Ile Ala Ile Asn Ala Gly Tyr Asp Gly Gly Val Val Val Asn Glu Val	
450 455 460 465	
GAA AAA CAC GAA GGG CAT TTT GGT TTT AAC GCT AGC AAT GGC AAG TAT	1948
Glu Lys His Glu Gly His Phe Gly Phe Asn Ala Ser Asn Gly Lys Tyr	
470 475 480	
GTG GAC ATG TTT AAA GAA GGC ATT ATT GAC CCC TTA AAA GTA GAA AGG	1996
Val Asp Met Phe Lys Glu Gly Ile Ile Asp Pro Leu Lys Val Glu Arg	
485 490 495	
ATC GCT TTA CAA AAT GCG GTT TCG GTT TCA AGC CTG CTT TTA ACC ACA	2044
Ile Ala Leu Gln Asn Ala Val Ser Val Ser Ser Leu Leu Leu Thr Thr	
500 505 510	
GAA GCC ACC GTG CAT GAA ATC AAA GAA GAA AAA GCG GCC CCA GCA ATG	2092
Glu Ala Thr Val His Glu Ile Lys Glu Glu Lys Ala Ala Pro Ala Met	
515 520 525	
CCT GAT ATG GGT GGC ATG GGC GGA ATG GGA GGC ATG GGC GGC ATG ATG	2140
Pro Asp Met Gly Gly Met Gly Gly Met Gly Gly Met Gly Gly Met Met	
530 535 540 545	
TAAGCCCCCT TGCTTTTGG TATCATCTGC TTTTAAATC CATCTTCTAG AATCCCCCT	2200
TCTAAAATCC CTTTTTGGG GGGTGCTTTT GGTGTTGATAA AACCGCTCGC TTTTAAAAAC	2260
GCGCAACAAA AAATCTGTGTT AAGC	2284

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 545 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Helicobacter pylori*

(ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..545
- (D) OTHER INFORMATION: /note= "Corresponds to figure 7A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Met	Ala	Lys	Glu	Ile	Lys	Phe	Ser	Asp	Ser	Ala	Arg	Asn	Leu	Leu	Phe	1	5	10	15
Glu	Gly	Val	Arg	Gln	Leu	His	Asp	Ala	Val	Lys	Val	Thr	Met	Gly	Pro	20	25	30	
Arg	Gly	Arg	Asn	Val	Leu	Ile	Gln	Lys	Ser	Tyr	Gly	Ala	Pro	Ser	Ile	35	40	45	
Thr	Lys	Asp	Gly	Val	Ser	Val	Ala	Lys	Glu	Ile	Glu	Leu	Ser	Cys	Pro	50	55	60	
Val	Ala	Asn	Met	Gly	Ala	Gln	Leu	Val	Lys	Glu	Asp	Ala	Ser	Lys	Thr	65	70	75	80
Ala	Asp	Ala	Ala	Gly	Asp	Gly	Thr	Thr	Thr	Ala	Thr	Val	Leu	Ala	Tyr	85	90	95	
Ser	Ile	Phe	Lys	Glu	Gly	Leu	Arg	Asn	Ile	Thr	Ala	Gly	Ala	Asn	Pro	100	105	110	
Ile	Glu	Val	Lys	Arg	Gly	Met	Asp	Lys	Ala	Pro	Glu	Ala	Ile	Ile	Asn	115	120	125	
Glu	Leu	Lys	Lys	Ala	Ser	Lys	Lys	Val	Gly	Gly	Lys	Glu	Glu	Ile	Thr	130	135	140	
Gln	Val	Ala	Thr	Ile	Ser	Ala	Asn	Ser	Asp	His	Asn	Ile	Gly	Lys	Leu	145	150	155	160
Ile	Ala	Asp	Ala	Met	Glu	Lys	Val	Gly	Lys	Asp	Gly	Val	Ile	Thr	Val	165	170	175	
Glu	Glu	Ala	Lys	Gly	Ile	Glu	Asp	Glu	Leu	Asp	Val	Val	Glu	Gly	Met	180	185	190	
Gln	Phe	Asp	Arg	Gly	Tyr	Leu	Ser	Pro	Tyr	Phe	Val	Thr	Asn	Ala	Glu	195	200	205	
Lys	Met	Thr	Ala	Gln	Leu	Asp	Asn	Ala	Tyr	Ile	Leu	Leu	Thr	Asp	Lys	210	215	220	
Lys	Ile	Ser	Ser	Met	Lys	Asp	Ile	Leu	Pro	Leu	Leu	Glu	Lys	Thr	Met	225	230	235	240
Lys	Glu	Gly	Lys	Pro	Leu	Leu	Ile	Ile	Ala	Glu	Asp	Ile	Glu	Gly	Glu	245	250	255	
Ala	Leu	Thr	Thr	Leu	Val	Val	Asn	Lys	Leu	Arg	Gly	Val	Leu	Asn	Ile	260	265	270	
Ala	Ala	Val	Lys	Ala	Pro	Gly	Phe	Gly	Asp	Arg	Arg	Lys	Glu	Met	Leu	275	280	285	
Lys	Asp	Ile	Ala	Val	Leu	Thr	Gly	Gly	Gln	Val	Ile	Ser	Glu	Glu	Leu	290	295	300	

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Gly Leu Ser Leu Glu Asn Ala Glu Val Glu Phe Leu Gly Lys Ala Lys
 305 310 315 320
 Ile Val Ile Asp Lys Asp Asn Thr Thr Ile Val Asp Gly Lys Gly His
 325 330 335
 Ser His Asp Val Lys Asp Arg Val Ala Gln Ile Lys Thr Gln Ile Ala
 340 345 350
 Ser Thr Thr Ser Asp Tyr Asp Lys Glu Lys Leu Gln Glu Arg Leu Ala
 355 360 365
 Lys Leu Ser Gly Gly Val Ala Val Ile Lys Val Gly Ala Ala Ser Glu
 370 375 380
 Val Glu Met Lys Glu Lys Lys Asp Arg Val Asp Asp Ala Leu Ser Ala
 385 390 395 400
 Thr Lys Ala Ala Val Glu Glu Gly Ile Val Ile Gly Gly Gly Ala Ala
 405 410 415
 Leu Ile Arg Ala Ala Gln Lys Val His Leu Asn Leu His Asp Asp Glu
 420 425 430
 Lys Val Gly Tyr Glu Ile Ile Met Arg Ala Ile Lys Ala Pro Leu Ala
 435 440 445
 Gln Ile Ala Ile Asn Ala Gly Tyr Asp Gly Gly Val Val Val Asn Glu
 450 455 460
 Val Glu Lys His Glu Gly His Phe Gly Phe Asn Ala Ser Asn Gly Lys
 465 470 475 480
 Tyr Val Asp Met Phe Lys Glu Gly Ile Ile Asp Pro Leu Lys Val Glu
 485 490 495
 Arg Ile Ala Leu Gln Asn Ala Val Ser Val Ser Ser Leu Leu Leu Thr
 500 505 510
 Thr Glu Ala Thr Val His Glu Ile Lys Glu Glu Lys Ala Ala Pro Ala
 515 520 525
 Met Pro Asp Met Gly Gly Met Gly Gly Met Gly Gly Met Gly Gly Met
 530 535 540
 Met
 545

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 118 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Helicobacter pylori*

(ix) FEATURE:

(A) NAME/KEY: Protein

(B) LOCATION: 1..118

(D) OTHER INFORMATION: /note= "Corresponds to figure 7B."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```

Met Lys Phe Gln Pro Leu Gly Glu Arg Val Leu Val Glu Arg Leu Glu
 1             5             10             15
Glu Glu Asn Lys Thr Ser Ser Gly Ile Ile Ile Pro Asp Asn Ala Lys
          20             25             30
Glu Lys Pro Leu Met Gly Val Val Lys Ala Val Ser His Lys Ile Ser
          35             40             45
Glu Gly Cys Lys Cys Val Lys Glu Gly Asp Val Ile Ala Phe Gly Lys
          50             55             60
Tyr Lys Gly Ala Glu Ile Val Leu Asp Gly Val Glu Tyr Met Val Leu
          65             70             75             80
Glu Leu Glu Asp Ile Leu Gly Ile Val Gly Ser Gly Ser Cys Cys His
          85             90             95
Thr Gly Asn His Asp His Lys His Ala Lys Glu His Glu Ala Cys Cys
          100             105             110
His Asp His Lys Lys His
          115

```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 591 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *H. felis*

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..591

(D) OTHER INFORMATION: /standard_name= "URE I"

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 1..591

(D) OTHER INFORMATION: /note= "Corresponds to figure 9."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ATG	TTA	GGT	CTT	GTG	TTA	TTG	TAT	GTT	GCG	GTC	GTG	CTG	ATC	AGC	AAC	48
Met	Leu	Gly	Leu	Val	Leu	Leu	Tyr	Val	Ala	Val	Val	Leu	Ile	Ser	Asn	
1				5					10					15		
GGA	GTT	AGT	GGG	CTT	GCA	AAT	GTG	GAT	GCC	AAA	AGC	AAA	GCC	ATC	ATG	96
Gly	Val	Ser	Gly	Leu	Ala	Asn	Val	Asp	Ala	Lys	Ser	Lys	Ala	Ile	Met	
			20					25					30			
AAC	TAC	TTT	GTG	GGG	GGG	GAC	TCT	CCA	TTG	TGT	GTA	ATG	TGG	TCG	CTA	144
Asn	Tyr	Phe	Val	Gly	Gly	Asp	Ser	Pro	Leu	Cys	Val	Met	Trp	Ser	Leu	
		35					40					45				
TCA	TCT	TAT	TCC	ACT	TTC	CAC	CCC	ACC	CCC	CCT	GCA	ACT	GGT	CCA	GAA	192
Ser	Ser	Tyr	Ser	Thr	Phe	His	Pro	Thr	Pro	Pro	Ala	Thr	Gly	Pro	Glu	
		50				55					60					
GAT	GTC	GCG	CAG	GTG	TCT	CAA	CAC	CTC	ATT	AAC	TTC	TAT	GGT	CCA	GCG	240
Asp	Val	Ala	Gln	Val	Ser	Gln	His	Leu	Ile	Asn	Phe	Tyr	Gly	Pro	Ala	
65				70					75					80		
ACT	GGT	CTA	TTG	TTT	GGT	TTT	ACC	TAC	TTG	TAT	GCT	GCC	ATC	AAC	AAC	288
Thr	Gly	Leu	Leu	Phe	Gly	Phe	Thr	Tyr	Leu	Tyr	Ala	Ala	Ile	Asn	Asn	
				85					90					95		
ACT	TTC	AAT	CTC	GAT	TGG	AAA	CCC	TAT	GGC	TGG	TAT	TGC	TTG	TTT	GTA	336
Thr	Phe	Asn	Leu	Asp	Trp	Lys	Pro	Tyr	Gly	Trp	Tyr	Cys	Leu	Phe	Val	
			100					105					110			
ACC	ATC	AAC	ACT	ATC	CCA	GCG	GCC	ATT	CTT	TCT	CAC	TAT	TCC	GAT	GCG	384
Thr	Ile	Asn	Thr	Ile	Pro	Ala	Ala	Ile	Leu	Ser	His	Tyr	Ser	Asp	Ala	
		115					120					125				
CTT	GAT	GAT	CAC	CGC	CTC	TTA	GGA	ATC	ACT	GAG	GGC	GAT	TGG	TGG	GCT	432
Leu	Asp	Asp	His	Arg	Leu	Leu	Gly	Ile	Thr	Glu	Gly	Asp	Trp	Trp	Ala	
	130					135					140					
TTC	ATT	TGG	CTT	GCT	TGG	GGT	GTT	TTG	TGG	CTC	ACT	GGT	TGG	ATT	GAA	480
Phe	Ile	Trp	Leu	Ala	Trp	Gly	Val	Leu	Trp	Leu	Thr	Gly	Trp	Ile	Glu	
145				150					155					160		
TGC	GCA	CTT	GGT	AAG	AGT	CTA	GGT	AAA	TTT	GTT	CCA	TGG	CTT	GCC	ATC	528
Cys	Ala	Leu	Gly	Lys	Ser	Leu	Gly	Lys	Phe	Val	Pro	Trp	Leu	Ala	Ile	
			165					170						175		
GTC	GAG	GGC	GTG	ATC	ACC	GCT	TGG	ATT	CCT	GCT	TGG	CTA	CTC	TTT	ATC	576
Val	Glu	Gly	Val	Ile	Thr	Ala	Trp	Ile	Pro	Ala	Trp	Leu	Leu	Phe	Ile	
			180					185						190		

CAA CAC TGG TCT TGA
Gln His Trp Ser
195

591

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 199 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: H. felis

(ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..199
- (D) OTHER INFORMATION: /note= "Corresponds to figure 10."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Lys	Gly	Trp	Met	Leu	Gly	Leu	Val	Leu	Leu	Tyr	Val	Ala	Val	Val	Leu	1	5	10	15
Ile	Ser	Asn	Gly	Val	Ser	Gly	Leu	Ala	Asn	Val	Asp	Ala	Lys	Ser	Lys	20	25	30	
Ala	Ile	Met	Asn	Tyr	Phe	Val	Gly	Gly	Asp	Ser	Pro	Leu	Cys	Val	Met	35	40	45	
Trp	Ser	Leu	Ser	Ser	Tyr	Ser	Thr	Phe	His	Pro	Thr	Pro	Pro	Ala	Thr	50	55	60	
Gly	Pro	Glu	Asp	Val	Ala	Gln	Val	Ser	Gln	His	Leu	Ile	Asn	Phe	Tyr	65	70	75	80
Gly	Pro	Ala	Thr	Gly	Leu	Leu	Phe	Gly	Phe	Thr	Tyr	Leu	Tyr	Ala	Ala	85	90	95	
Ile	Asn	Asn	Thr	Phe	Asn	Leu	Asp	Trp	Lys	Pro	Tyr	Gly	Trp	Tyr	Cys	100	105	110	
Leu	Phe	Val	Thr	Ile	Asn	Thr	Ile	Pro	Ala	Ala	Ile	Leu	Ser	His	Tyr	115	120	125	
Ser	Asp	Ala	Leu	Asp	Asp	His	Arg	Leu	Leu	Gly	Ile	Thr	Glu	Gly	Asp	130	135	140	
Trp	Trp	Ala	Phe	Ile	Trp	Leu	Ala	Trp	Gly	Val	Leu	Trp	Leu	Thr	Gly	145	150	155	160
Trp	Ile	Glu	Cys	Ala	Leu	Gly	Lys	Ser	Leu	Gly	Lys	Phe	Val	Pro	Trp	165	170	175	

Leu Ala Ile Val Glu Gly Val Ile Thr Ala Trp Ile Pro Ala Trp Leu
 180 185 190

Leu Phe Ile Gln His Trp Ser
 195

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: unknown
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:
 (A) ORGANISM: H. pylori

(ix) FEATURE:
 (A) NAME/KEY: Protein
 (B) LOCATION: 1..27
 (D) OTHER INFORMATION: /note= "Corresponds to sequence mentioned at page 13 of the description."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Gly Ser Cys Cys His Thr Gly Asn His Asp His Lys His Ala Lys Glu
 1 5 10 15
 His Glu Ala Cys Cys His Asp His Lys Lys His
 20 25

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
 (A) NAME/KEY: misc feature
 (B) LOCATION: 1..27
 (D) OTHER INFORMATION: /note= "Corresponds to primer 1F of table 2 (page 51)."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CAUCCNAARG ARYTNGAYAA RYTNATC

27

(2) INFORMATION FOR SEQ ID NO: 1

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

MOLECULE TYPE: DNA (genomic)

FEATURE:

NAME/KEY: misc feature

LOCATION: 1..30

(D) OTHER INFORMATION: /note= "Corresponds to primer 1R of table 2 (page 51)."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

YTCYTTNCGN GGNSWDATYT TYTTCATCUA

30

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: misc feature

(B) LOCATION: 1..38

(D) OTHER INFORMATION: /note= "Corresponds to primer 2F of table 2 (page 51 of the description)."

(ix) FEATURE:

(A) NAME/KEY: misc feature

(B) LOCATION: 6..11

(D) OTHER INFORMATION: /note= "Restriction site introduced in the amplified fragment (EcoRI)."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CCGGAGAATT CATTAGCAGA AAAGAATATG TTTCTATG

38

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(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..38
- (D) OTHER INFORMATION: /note= "Corresponds to primer 2R of table 2 (page 51)."

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 6..11
- (D) OTHER INFORMATION: /note= "Restriction site introduced in the amplified fragment (PstI)."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

ACGTTCTGCA GCTTACGAAT AACTTTTGTT GCTTGAGC

38

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..20
- (D) OTHER INFORMATION: /note= "Corresponds to primer 3F of table 2 (page 51)."

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..6
- (D) OTHER INFORMATION: /note= "Restriction site introduced in the amplified fragment (BamHI)."

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GGATCCAAAA AGATTTCACG

20

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 30 base pairs
 - (E) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..30
- (D) OTHER INFORMATION: /note= "Corresponds to primer 3R of table 2 (page 51)."

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 3..8
- (D) OTHER INFORMATION: /note= "Restriction site introduced in the amplified fragment (HindIII)"

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 9..14
- (D) OTHER INFORMATION: /note= "Restriction site introduced in the amplified fragment (PstI)."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GGAAGCTTCT GCAGGTGTGC TTCCCCAGTC

30

(2) INFORMATION FOR SEQ ID NO: 16:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 38 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..38
- (D) OTHER INFORMATION: /note= "Corresponds to oligo 1 of page 69."

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 6..11
- (D) OTHER INFORMATION: /note= "restriction site EcoRI"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCGGAGAATT CAAGTTTCAA CCATTAGGAG AAAGGGTC

38

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..38
- (D) OTHER INFORMATION: /note= "Corresponds to oligo 2 of page 69."

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 6..11
- (D) OTHER INFORMATION: /note= "Restriction site PstI."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

ACGTTCTGCA GTTTAGTGTT TTTGTGATC ATGACAGC

38

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..38
- (D) OTHER INFORMATION: /note= "Corresponds to oligo 3 of page 69."

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 6..11
- (D) OTHER INFORMATION: /note= "Restriction site EcoRI."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CCGGAGAATT CGCAAAGAA ATCAAATTTT CAGATAGC

38

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 1..38
- (D) OTHER INFORMATION: /note= "Corresponds to oligo 4 of page 69."

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: 6..11
- (D) OTHER INFORMATION: /note= "Restriction site PstI."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

ACGTTCTGCA GATGATACCA AAAAGCAAGG GGGCTTAC

38

CLAIMS

1. A composition comprising a mixture of antigens of Helicobacter wherein said mixture consists essentially of UreB and HspA of H. pylori or polypeptides having at least 75 % and preferably 80 to 90 % similarity with
5 said UreB or HspA, or fragments thereof capable of eliciting antibodies recognized by H. pylori, or an immune cellular response against an H. pylori infection .
2. A composition according to Claim 1 wherein said mixture consists essentially of UreB and HspA of H. felis or polypeptides having at least
10 75% and preferably 80 to 90 % similarity with said UreB or HspA, or fragments thereof capable of inducing antibodies recognized by H. pylori or an immune cellular response against an H. pylori infection.
3. A composition according to claim 1 or claim 2, wherein said HspA fragment is the C-terminal sequence of HspA of H. pylori replying to or
15 included in the following amino-acid sequence :
- G S C C H T G N H D H K H A K E H E A C C H D H K K H .
4. A composition according to anyone of claims 1 to 3 wherein the
20 amount of antigens or polypeptides having at least 75 % and preferably 80 to 90 % similarity with said UreB or HspA, or fragments thereof is sufficient to elicit an immune response in a host to whom it is administered.
5. A composition according to anyone of claims 1 to 4 wherein the fragments comprise between 9 and 200 aminoacid residues and are in an
25 amount sufficient to elicit an immune response in a host to whom it is administered
6. A composition according to anyone of claims 1 to 5, characterized in that the antigens are recombinant antigens.

7. A composition according to anyone of claims 1 to 6, characterized in that the antigens and/or fragments thereof are included in fusion proteins.

8. A composition according to anyone of claims 1 to 5, which is
5 substantially free of UreA.

9. A composition according to anyone of claims 1 to 6, which is substantially free of other H. pylori or H. felis antigens.

10. A composition according to anyone of claims 1 to 9, which further contains an adjuvant.

10 11. A composition according to anyone of claims 1 to 10, which contains a mucosal adjuvant, for example, cholera or E. coli holotoxins.

12. Pharmaceutical composition, characterized in that it comprises, as active ingredient, a composition according to anyone of claims 1 to 10, in combination with physiologically acceptable excipient(s).

15 13. Composition according to anyone of claims 1 to 11 or pharmaceutical composition according to claim 12, for use as vaccine in protecting an animal or a human host against Helicobacter infection.

14. Composition according to anyone of claims 1 to 11 or pharmaceutical composition according to claim 12, for use as therapeutic
20 or curative treatment of an animal or human host against Helicobacter infection.

15. Composition according to anyone of claims 1 to 11 or pharmaceutical composition according to claim 12, for inducing or enhancing a protective response against mucosal infection by Helicobacter
25 pylori in a host to whom it is administered.

16. Use of H. pylori Hsp antigens for example HspA or HspB, or polypeptides having at least 75 % and preferably 80 to 90 % similarity with said UreB or HspA, or fragments thereof for eliciting a protective response